



(11)

EP 3 496 757 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
08.07.2026 Bulletin 2026/28

(21) Application number: **17840397.8**

(22) Date of filing: **12.08.2017**

(51) International Patent Classification (IPC):

A61K 9/12 (2006.01)	A61K 45/06 (2006.01)
A61P 35/00 (2006.01)	A61K 31/517 (2006.01)
A61K 31/519 (2006.01)	A61K 47/64 (2017.01)
A61P 19/02 (2006.01)	C07K 16/28 (2006.01)
A61K 47/68 (2017.01)	A61K 47/69 (2017.01)

(52) Cooperative Patent Classification (CPC):
(C-Sets available)

**A61P 35/00; A61K 9/1271; A61K 9/1277;
A61K 31/517; A61K 31/519; A61K 45/06;
A61K 47/645; A61K 47/6849; A61K 47/6913;
A61P 19/02; C07K 16/28; A61K 2300/00;
C07K 2317/73; C07K 2317/76; C07K 2317/92;**

(Cont.)

(86) International application number:
PCT/US2017/046667

(87) International publication number:
WO 2018/031980 (15.02.2018 Gazette 2018/07)

(54) **POLYGLUTAMATED ANTIFOLATES AND USES THEREOF**

POLYGLUTAMAT-ANTIFOLATE UND VERWENDUNGEN DAVON

ANTIFOLATES DE POLYGLUTAMATES ET LEURS UTILISATIONS.

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

(30) Priority: **12.08.2016 US 201662374458 P**
11.08.2017 US 201715675695
11.08.2017 US 201715675701

(43) Date of publication of application:
19.06.2019 Bulletin 2019/25

(73) Proprietor: **L.E.A.F Holdings Group LLC**
Gulph Mills, PA 19428 (US)

(72) Inventors:
• **NIYIKIZA, Clet**
Gulph Mills, PA 19428 (US)
• **MOYO, Victor Mandla**
Ringoes, NJ 08551 (US)

(74) Representative: **FRKelly**
Waterways House
Grand Canal Quay
Dublin D02 PD39 (IE)

(56) References cited:
WO-A1-01/05405 WO-A1-2006/074416
WO-A1-2014/046630 WO-A2-2005/070465
WO-A2-2006/002049 WO-A2-2007/098089
RU-C2- 2 423 114 US-A- 4 584 375
US-A1- 2009 155 345 US-A1- 2013 259 922
US-A1- 2014 086 939 US-A1- 2015 239 956

- **NING ; HU ET AL: "Lipid-based dispersions for drug delivery", WO2005070465 A2, 1 January 2005 (2005-01-01), pages 1 - 2, XP055668142**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 3 496 757 B1

- ROLAND J. W. MEESTERS ET AL: "Assessment of intracellular methotrexate and methotrexate-polyglutamate metabolite concentrations in erythrocytes by ultrafast matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry : MTX-polyglutamate concentration in erythrocytes", RAPID COMMUNICATIONS IN MASS SPECTROMETRY., vol. 25, no. 20, 30 October 2011 (2011-10-30), GB, pages 3063 - 3070, XP055666549, ISSN: 0951-4198, DOI: 10.1002/rcm.5202
 - A L JACKMAN ET AL: "ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study", CANCER RESEARCH, 1 January 1991 (1991-01-01), Germany, pages 5579 - 5586, XP055666559
 - DATABASE BIOSIS [online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; JACKMAN A L ET AL: "ICI-D1694 A QUINAZOLINE ANTIFOLATE THYMIDYLATE SYNTHASE INHIBITOR THAT IS A POTENT INHIBITOR OF L1210 TUMOR CELL GROWTH IN-VITRO AND IN-VIVO A NEW AGENT FOR CLINICAL STUDY", XP002341574, retrieved from BIOSIS Database accession no. PREV199293007206
 - C. PITTS ET AL: "Interaction Energy Analyses of Folate Analog Binding to Human Dihydrofolate Reductase: Contribution of the Antifolate Substructural Regions to Complex Stability", DRUG METABOLISM AND DRUG INTERACTIONS., vol. 16, no. 2, 1 January 2000 (2000-01-01), IL, XP055666556, ISSN: 0792-5077, DOI: 10.1515/DMDI.2000.16.2.99
 - NITZAN GONEN ET AL: "Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance", DRUG RESISTANCE UPDATES, vol. 15, no. 4, 1 August 2012 (2012-08-01), GB, pages 183 - 210, XP055668141, ISSN: 1368-7646, DOI: 10.1016/j.drug.2012.07.002
 - KA-YUN NG ET AL: "Liposome-dependent delivery of pteridine antifolates: a two-compartment growth inhibition assay for evaluating drug leakage and metabolism", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - BIOMEMBRANES, ELSEVIER, AMSTERDAM, NL, vol. 981, no. 2, 6 June 1989 (1989-06-06), pages 261 - 268, XP023574621, ISSN: 0005-2736, [retrieved on 19890606], DOI: 10.1016/0005-2736(89)90036-9
 - ANONYMOUS: "Antifolate - Wikipedia", WIKIPEDIA, 29 December 2020 (2020-12-29), pages 1 - 5, XP055790636, Retrieved from the Internet <URL:https://en.wikipedia.org/wiki/Antifolate> [retrieved on 20210329]
 - ELDIN NOHA ESSAM ET AL: "Encapsulation in a rapid-release liposomal formulation enhances the anti-tumor efficacy of pemetrexed in a murine solid mesothelioma-xenograft model", EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES, ELSEVIER AMSTERDAM, NL, vol. 81, 28 September 2015 (2015-09-28), pages 60 - 66, XP029319401, ISSN: 0928-0987, DOI: 10.1016/J.EJPS.2015.09.015
- (52) Cooperative Patent Classification (CPC): (Cont.)
Y02A 50/30
- C-Sets
A61K 31/519, A61K 2300/00

Description

BACKGROUND

[0001] This disclosure generally relates to L-gamma polyglutamated antifolate compositions, including delivery vehicles such as targeted and untargeted liposomes filled with the polyglutamated antifolates, and methods of making the compositions for use in treating diseases including hyperproliferative diseases such as cancer, disorders of the immune system such as rheumatoid arthritis and infectious disease such as HIV.

[0002] Folates are indispensable for cell growth and tissue regeneration. Mammalian cells do not synthesize folates *de novo* and rely on extracellular folates taken up by three major folate uptake systems: a reduced folate carrier (RFC) system; a system of folate receptors (FRs) α and β ; and a folate symporter (PCFT) system. Antifolates that target folate-dependent biosynthetic pathways function as anti-proliferative agents. Naturally occurring folates exist within cells as polyglutamates through the action of the enzyme folylpolyglutamyl synthetase (FPGS), which may add up to 6 glutamyl groups in a gamma peptide linkage to the folate substrate. Polyglutamation serves at least 3 main purposes: (1) it facilitates the accumulation of intracellular folates in vast excess of the monoglutamate pool, which is freely transportable into and out of cells; (2) it allows selective intracellular retention of these relatively large anionic molecules; and (3) it greatly enhances folate cofactor affinity for several folate-dependent enzymes, including thymidylate synthase and AICAR transformylase (see, e.g., FIG. 2).

[0003] Antifolates are a class of antiproliferatives that were first developed more than 70 years ago as "folic acid mimic molecule" cytotoxic agents. The rationale was to design a class of molecules that would counter the action of folic acid in fast replicating cells such as cancer cells, taking advantage of physiological folate transport mechanism and their facilitative intracellular mode of action for DNA replication during the cell division. Specifically, antifolates were designed to mimic folic acid in its systemic transport, physiologic cell uptake (e.g., via reduced folate carriers (RFCs) and proton-coupled folate transporters (PCFTs)) and intracellular processing. Antifolates act specifically during DNA and RNA synthesis, exerting a cytotoxic effect during the S-phase of the cell cycle. As a result, they have a greater toxic effect on rapidly dividing cells such as malignant and myeloid cells.

[0004] Antifolates are widely recognized for their inhibition of folate metabolism. Major antifolate enzyme targets and example antifolates that target these enzymes include: (a) dihydrofolate reductase (DHFR) [e.g., methotrexate (MTX) and pralatrexate], (b) thymidylate synthase (TS) [e.g., raltitrexed (RTX), GW1843U, and pemetrexed (PMX)], (c) β -glycinamide ribonucleotide formyl transferase (GARFTase) [e.g., lometrexol (LMX), PMX] and (d) 5-aminoimidazole-4-carboxamide ribonucleotide formyl transferase (AICARFTase) [e.g., PMX]. Inhibition of the above enzymes suppresses *de novo* nucleotide biosynthesis, resulting in an imbalance of purine and pyrimidine precursors and rendering cells incapable of undergoing accurate DNA replication, ultimately resulting in cell death. Accordingly, it is not surprising that inhibitors of the folate metabolism pathway play important roles in treating hyperproliferative diseases including hematologic malignancies and solid tumors, as well as disorders of the immune system such as rheumatoid arthritis.

[0005] Each of the above antifolates is transported by the reduced folate carrier (RFC), which is the main transport system at physiologic pH. RFC is ubiquitously expressed in normal and diseased cells, and consequently these drugs suffer from dose-limiting toxicity which is a major obstacle in the chemotherapy of cancer.

[0006] Pemetrexed (sold commercially under the brand name ALIMTA®) is a antifolate containing a 6-5 fused pyrrolo [2,3,-d]pyrimidine nucleus that inhibits thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase (GARFT), and dihydrofolate reductase (DHFR), folate-dependent enzymes involved in the synthesis of thymidine and purine nucleotides. Like methotrexate, pemetrexed is transported into cells by the RFC and membrane folate-binding proteins, where it is polyglutamated by folylpoly-gamma-glutamate synthetase. Polyglutamated forms of pemetrexed have greater intracellular retention and have greater affinity for TS and GARFT compared to pemetrexed monoglutamate. Pemetrexed is approved for the treatment of mesothelioma and non-small lung carcinoma (NSCLC). Myelosuppression is typically the dose-limiting toxicity with pemetrexed therapy and has limited the clinical applications of this drug. Pretreatment with folic acid and vitamin B is now used to ameliorate the most frequent side effects that include bone marrow suppression, fatigue, and skin rash.

[0007] One of the challenges of cancer treatment is delivering cytotoxic agents to cancer cells while minimizing and/or reducing the effect of such agents on normal healthy cells. To address the toxicity of antifolates in normal cells, WO 2016/25882 describes liposomal formulations of antifolates that are targeted to cancer cells using, for example, antibodies having a specific affinity for folate receptors expressed by many cancer cells. This formulation can reduce and/or minimize the effects of the antifolates on healthy cells, meaning that patients can experience fewer side effects. WO0105405 relates to the use of folylpolyglutamyl synthetase (FPGS) gene transfer to enhance the sensitivity of several types of tumor cells to polyglutamylatable antifolate drugs, such as methotrexate (MTX) and edatrexate (EDX). WO2006002049 provides methods for determining a level of methotrexate polyglutamates (MTXPGs) in an individual undergoing methotrexate (MTX) therapy and for optimizing dose efficacy of MTX therapy in an individual by genotyping the individual at a polymorphic site in at least one folate pathway gene. WO2007098089 relates to methotrexate and analog N-oxides

having activity for treating hyperproliferative disorders. Further, the invention relates to pharmaceutical compositions and methods of using methotrexate and analog N-oxides, alone or in combination with one or more other active agents or treatments, to treat hyperproliferative disorders. WO2005070465 provides lipid-based dispersion comprising comprising, a) phosphatidyl choline; b) an anionic phospholipid; optionally c) up to 1% cholesterol by weight of total lipids; and optionally d) a therapeutic agent; wherein the mean particle size measured by dynamic light scattering is less than 100 nm. The invention also provides pharmaceutical compositions comprising such a dispersion as well as methods of producing a therapeutic effect in a mammal comprising administering an effective amount of such a dispersion. Meesters et al. RAPID COMMUNICATIONS IN MASS SPECTROMETRY., vol. 25, 3063-3070, 2011 relates to an ultrafast quantitative and high-throughput mass spectrometric method using matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry for determination of intracellular erythrocyte concentrations of the antifolate drug methotrexate (MTX) and its polyglutamate metabolites. WO2006074416 provides a method for increasing the efficacy of antifolates which act via inhibition of dihydrofolate reductase (DHFR). Jackman et al. CANCER RESEARCH, 1991, pages 5579-5586 relates to a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo. Pitts et al. DRUG METABOLISM AND DRUG INTERACTIONS., vol. 16, 99-121, 2000 relates to the three-dimensional structure of the human dihydrofolate reductase (DHFR), methotrexate tetrazole, and NADPH ternary complex was used to model the corresponding ternary complexes with methotrexate tetrazole replaced by methotrexate, methotrexate-polyglutamate with three glutamyl residues, and 5,10-deazaaminopterin, respectively. Gonen et al. DRUG RESISTANCE UPDATES, vol. 15, 183-210, 2012 relates to antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Ng et al. BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - BIOMEMBRANES, vol. 981, 261-268, 1989 relates to a two-compartment growth inhibition assay that can provide information about leakage, metabolism and delivery of liposome-dependent drugs under cell culture conditions, and at drug concentrations that are relevant to drug delivery. RU2423114 relates to compositions containing methoxyamine and an antimetabolic anticancer agent, and methods for treating certain cancers by administration of such compositions. Eldin et al. EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 81, 60-66, 2015 relates to a PEG-coated liposome encapsulating the anti-folate drug pemetrexed (PMX).

[0008] Despite the advances in treating cancer and other hyperproliferative disorders using antifolates, additional compositions and methods are needed for improving the efficacy and decreasing the dose-limiting toxicity associated with antifolate therapy. The present disclosure provides compositions and methods that address these needs.

BRIEF SUMMARY

[0009] This disclosure generally relates polyglutamated antifolate compositions, including delivery vehicles such as liposomes filled with the polyglutamated antifolates, and methods of making the compositions for use in treating diseases including hyperproliferative diseases such as cancer, disorders of the immune system such as rheumatoid arthritis and infectious disease such as HIV. This disclosure further relates to pentaglutamated and hexaglutamated antifolate compositions, including delivery vehicles such as liposomes filled with the pentaglutamated and hexaglutamated antifolates, and methods of making the compositions for use in treating diseases including hyperproliferative diseases, disorders of the immune system and infectious disease.

[0010] The references to methods of treatment in the subsequent paragraphs of this description are to be interpreted as references to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human body by therapy

[0011] The present invention relates to a pegylated liposomal polyglutamated antifolate (LPA) composition comprising a pentaglutamated or hexaglutamated polyglutamated antifolate comprising L-glutamates linked by their gamma carboxyl groups, wherein the polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated GW1843, polyglutamated aminopterin, polyglutamated LY309887; optionally wherein the polyglutamated antifolate is polyglutamated PMX, MTX, RTX, or LTX.

[0012] By way of example and without limitation, the disclosure describes liposome compositions that contain polyglutamated (e.g., pentaglutamated and hexaglutamated) forms of the antifolates MTX, PMX, LTX, AG2034, RTX, piritrexim, pralatrexate, AG2034, GW1843, aminopterin, and LY309887. These compositions provide improvements to the efficacy and safety of delivering antifolates to cancer cells by providing the preferential delivery of a more cytotoxic payload (e.g., polyglutamated antifolates) compared to the cytotoxicity of antifolate in its administered monoglutamated state. The disclosure also provides targeted liposome compositions that contain a targeting moiety having a specific affinity for an epitope (antigen) expressed on the surface of a target cell on interest. The targeted liposomes provide further improvements to the efficacy and safety of delivering antifolates to cancer cells by specifically delivering polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolates to the target cell.

[0013] The disclosure provides a composition comprising a polyglutamated antifolate (e.g., pentaglutamated or

hexaglutamated). The composition may comprise pentaglutamated antifolate. The composition may comprise hexaglutamated antifolate. The polyglutamated antifolate may be one or more members selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further

embodiments, the polyglutamated member is pentaglutamated. The polyglutamated member may be hexaglutamated. **[0014]** In one embodiment, the composition comprises polyglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the composition comprises pentaglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the composition comprises hexaglutamated PMX, MTX, RTX, and/or LTX.

[0015] In one embodiment, the composition comprises polyglutamated PMX. In a further embodiment the composition comprises pentaglutamated PMX. In a further embodiment the composition comprises hexaglutamated PMX.

[0016] In another embodiment, the composition comprises polyglutamated MTX. In a further embodiment the composition comprises pentaglutamated MTX. In a further embodiment the composition comprises hexaglutamated MTX.

[0017] In another embodiment, the composition comprises polyglutamated RTX. In a further embodiment the composition comprises pentaglutamated RTX. In a further embodiment the composition comprises hexaglutamated RTX.

[0018] In an additional embodiment, the composition comprises polyglutamated LTX. In a further embodiment the composition comprises pentaglutamated LTX. In a further embodiment the composition comprises hexaglutamated LTX.

[0019] The disclosure provides a liposomal polyglutamated antifolate (LPA) composition that comprises a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate. For example, the polyglutamated antifolate may be in a HEPES buffered solution within a liposome. According to the invention, the LPA composition is pegylated (PLPA). In some embodiments, the PLPA composition comprises a pentaglutamated antifolate. In some embodiments, the PLPA composition comprises a hexaglutamated antifolate. In some embodiments, the PLPA liposome is anionic or neutral. In other embodiments, the PLPA liposome is cationic. In some embodiments, the PLPA composition comprises at least 10%, at least 20%, or at least 30% liposome entrapped polyglutamated antifolate. In some embodiments, the PLPA liposomes have a diameter in the range of 20 nm to 200 nm, 30 nm to 175 nm, or 50 nm to 150 nm. In some embodiments, the PLPA liposomes have a diameter in the range of 30 nm to 175 nm or 50 nm to 150 nm. In further embodiments, the PLPA liposomes have a diameter in the range of 80 nm to 120 nm.

[0020] In some embodiments, the PLPA composition comprises a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the polyglutamated antifolate is pentaglutamated. In further embodiments, the polyglutamated antifolate is hexaglutamated. In one embodiment, the PLPA composition comprises polyglutamated PMX, MTX, RTX, or LTX. In a further embodiment the PLPA composition comprises pentaglutamated PMX, MTX, RTX, or LTX. In a further embodiment the PLPA composition comprises hexaglutamated PMX, MTX, RTX, or LTX. In one embodiment, the PLPA composition comprises polyglutamated PMX. In a further embodiment the PLPA composition comprises pentaglutamated PMX. In a further embodiment the PLPA composition comprises hexaglutamated PMX.

[0021] In another embodiment, the PLPA composition comprises polyglutamated MTX. In a further embodiment the PLPA composition comprises pentaglutamated MTX. In a further embodiment the PLPA composition comprises hexaglutamated MTX.

[0022] In another embodiment, the PLPA composition comprises polyglutamated RTX. In a further embodiment the PLPA composition comprises pentaglutamated RTX. In a further embodiment the PLPA composition comprises hexaglutamated RTX.

[0023] In an additional embodiment, the PLPA composition comprises polyglutamated LTX. In a further embodiment the PLPA composition comprises pentaglutamated LTX. In a further embodiment the PLPA composition comprises hexaglutamated LTX.

[0024] The invention provides a liposomal polyglutamated antifolate composition wherein the liposome is pegylated and comprises a polyglutamated antifolate and a targeting moiety attached to one or both of a PEG and the exterior of the liposome, and wherein the targeting moiety has a specific affinity for a surface antigen on a target cell of interest. In some embodiments, the targeting moiety is a polypeptide. In further embodiments, the targeting moiety is an antibody or a fragment of an antibody. In additional embodiments, the targeting moiety comprises one or more of an antibody, a humanized antibody, an antigen binding fragment of an antibody, a single chain antibody, a single-domain antibody, a bi-specific antibody, a synthetic antibody, a pegylated antibody, and a multimeric antibody. In additional embodiments, the targeting moiety has the specific affinity for an epitope on a tumor cell surface antigen that is present on a tumor cell but absent or inaccessible on a non-tumor cell. In some embodiments, the targeting moiety-PLPA further comprises one or more of an immunostimulatory agent, a detectable marker and a maleimide disposed on at least one of the PEG and the exterior of the liposome. In some embodiments, the targeting moiety-PLPA liposome is anionic or neutral. In other embodiments, the targeting moiety-PLPA liposome is cationic. In some embodiments, the targeting moiety-PLPA composition comprises at least 10% liposome entrapped polyglutamated antifolate. In additional embodiments, the

targeting moiety-PLPA liposomes have a diameter in the range of 20 nm to 200 nm. In further embodiments, the liposomes have a diameter in the range of 80 nm to 120 nm.

[0025] In some embodiments, the targeting moiety-PLPA comprises a polypeptide targeting moiety such as an antibody or an antibody fragment and the targeting moiety binds a target antigen with an equilibrium dissociation constant (K_d) in a range of 0.5×10^{-10} to 10×10^{-6} as determined using BIACORE analysis. In further embodiments, the targeting moiety comprises a polypeptide that specifically binds a folate receptor. In some embodiments the folate receptor bound by the targeting moiety is one or more folate receptors selected from the group consisting of: folate receptor alpha (FR- α), folate receptor beta (FR- β), and folate receptor delta (FR- δ).

[0026] In additional embodiments, the invention provides a liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate (LPA) composition and/or a pegylated-LPA (PLPA) composition for use in a method for treating cancer, the use comprising administering an effective amount of the liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate (LPA) composition and/or the pegylated-LPA (PLPA) composition to a subject having or at risk of having cancer. In some embodiments, the method is administered to treat a cancer selected from the group consisting of: lung cancer, pancreatic, breast cancer, ovarian cancer, lung cancer, prostate cancer, head and neck cancer, gastric cancer, gastrointestinal cancer, colon cancer, esophageal cancer, cervical cancer, kidney cancer, biliary duct cancer, gallbladder cancer, and a hematologic malignancy. In some embodiments, the administered polyglutamated antifolate is one or more members selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the administered composition comprises pentaglutamated antifolates. In further embodiments, the administered composition comprises hexaglutamated antifolates. In some embodiments, the administered composition comprises the polyglutamated form of PMX, MTX, RTX, and/or LTX. In further embodiments, the administered composition comprises the pentaglutamated form of PMX, MTX, RTX, and/or LTX. In further embodiments, the administered composition comprises the hexaglutamated form of PMX, MTX, RTX, and/or LTX. In one embodiment, the administered composition comprises the pentaglutamated form of PMX. In one embodiment, the administered composition comprises the hexaglutamated form of PMX. In another embodiment, the administered composition comprises the pentaglutamated form of MTX. In another embodiment, the administered composition comprises the hexaglutamated form of MTX. In another embodiment, the administered composition comprises the pentaglutamated form of RTX. In another embodiment, the administered composition comprises the hexaglutamated form of RTX. In an additional embodiment, the administered composition comprises the pentaglutamated form of LTX. In an additional embodiment, the administered composition comprises the hexaglutamated form of LTX.

[0027] The invention provides a pegylated liposomal polyglutamated antifolate (LPA) composition comprising a pentaglutamated or hexaglutamated polyglutamated antifolate for use in a method for treating cancer, the use comprising administering an effective amount of the composition to a subject having or at risk of having cancer. In some embodiments, the method is administered to treat a cancer selected from the group consisting of: lung cancer, pancreatic, breast cancer, ovarian cancer, lung cancer, prostate cancer, head and neck cancer, gastric cancer, gastrointestinal cancer, colon cancer, esophageal cancer, cervical cancer, kidney cancer, biliary duct cancer, gallbladder cancer, and a hematologic malignancy. In some embodiments, the administered polyglutamated antifolate is one or more members selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the administered composition comprises pentaglutamated antifolates. In further embodiments, the administered composition comprises hexaglutamated antifolates. In some embodiments, the administered composition comprises the polyglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the pentaglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the hexaglutamated form of PMX, MTX, RTX, and/or LTX. In a further embodiment, the administered composition comprises the pentaglutamated form of PMX. In a further embodiment, the administered composition comprises the hexaglutamated form of PMX. In another further embodiment, the administered composition comprises the pentaglutamated form of MTX. In another further embodiment, the administered composition comprises the hexaglutamated form of MTX. In another further embodiment, the administered composition comprises the pentaglutamated form of RTX. In another further embodiment, the administered composition comprises the hexaglutamated form of RTX. In an additional further embodiment, the administered composition comprises the pentaglutamated form of LTX. In an additional further embodiment, the administered composition comprises the hexaglutamated form of LTX.

[0028] In some embodiments, the invention provides a LPA composition and/or a pegylated-LPA (PLPA) composition to a subject having or at risk of having cancer, wherein the PLPA and/or LPA composition further contains a targeting moiety having a specific affinity for a surface antigen (epitope) on the cancer for use in a method of treating cancer, the use comprising administering an effective amount of the LPA composition and/or the pegylated-LPA (PLPA) composition to a

subject having or at risk of having cancer. In further embodiments, the disclosure provides a method for treating cancer that comprises administering a liposomal polyglutamated antifolate composition that comprises a polyglutamated antifolate and a targeting moiety that has a specific binding affinity for a folate receptor to a subject having or at risk of having cancer expresses on its surface the folate receptor bound by the targeting moiety. In further embodiments, the targeting moiety has a specific binding affinity for folate receptor alpha (FR- α), folate receptor beta (FR- β), and/or folate receptor delta (FR- δ).

[0029] In some embodiments, the method is administered to treat a cancer selected from the group consisting of: lung cancer, pancreatic, breast cancer, ovarian cancer, lung cancer, prostate cancer, head and neck cancer, gastric cancer, gastrointestinal cancer, colon cancer, esophageal cancer, cervical cancer, kidney cancer, biliary duct cancer, gallbladder cancer, and a hematologic malignancy. In some embodiments, the administered polyglutamated antifolate is one or more members selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the administered composition comprises pentaglutamated antifolates. In further embodiments, the administered composition comprises hexaglutamated antifolates. In some embodiments, the administered composition comprises the polyglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the pentaglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the hexaglutamated form of PMX, MTX, RTX, and/or LTX. In a further embodiment, the administered composition comprises the pentaglutamated form of PMX. In a further embodiment, the administered composition comprises the hexaglutamated form of PMX. In another further embodiment, the administered composition comprises the pentaglutamated form of MTX. In another further embodiment, the administered composition comprises the hexaglutamated form of MTX. In another further embodiment, the administered composition comprises the pentaglutamated form of RTX. In another further embodiment, the administered composition comprises the hexaglutamated form of RTX. In an additional further embodiment, the administered composition comprises the pentaglutamated form of LTX. In an additional further embodiment, the administered composition comprises the hexaglutamated form of LTX.

[0030] In additional embodiments, the invention provides a liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate composition that comprises a liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate containing a targeting moiety that has a specific affinity for a surface antigen on the immune cell of interest wherein the liposome is pegylated for use in a method for treating a disorder of the immune system, the use comprising administering an effective amount the liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate composition to a subject having or at risk of having an immune system disorder. In some embodiments, the method is administered to treat an autoimmune disease. In a further embodiment the method is administered to treat rheumatoid arthritis. In some embodiments, the administered polyglutamated antifolate is one or more members selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the administered composition comprises pentaglutamated antifolates. In further embodiments, the administered composition comprises hexaglutamated antifolates. In some embodiments, the administered composition comprises the polyglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the pentaglutamated form of PMX, MTX, RTX, and/or LTX. In some embodiments, the administered composition comprises the pentaglutamated form of PMX, MTX, RTX, and/or LTX. In another further embodiment, the administered composition comprises the hexaglutamated form of MTX. In a further embodiment, the administered composition comprises the pentaglutamated form of PMX. In a further embodiment, the administered composition comprises the hexaglutamated form of PMX. In another further embodiment, the administered composition comprises the pentaglutamated form of RTX. In another further embodiment, the administered composition comprises the hexaglutamated form of RTX. In an additional further embodiment, the administered composition comprises the pentaglutamated form of LTX. In an additional further embodiment, the administered composition comprises the hexaglutamated form of LTX.

[0031] In additional embodiments the disclosure provides a method of preparing a composition that comprises a liposomal polyglutamated antifolate composition, the method comprising: forming a mixture comprising: liposomal components; polyglutamated antifolate in solution; homogenizing the mixture to form liposomes in the solution; and processing the mixture to form liposomes containing polyglutamated antifolate.

[0032] Pharmaceutical compositions comprising a liposomal polyglutamated (e.g., pentaglutamated and/or hexaglutamated) antifolate (LPA) composition and/or a pegylated-LPA (PLPA) composition and optionally further comprising a targeting moiety that has a specific affinity for a surface antigen on the surface of a target cell of interest are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0033]

FIG. 1 depicts homeostasis of folates and cellular accumulation of antifolates. Influx and efflux (anti)folates transporters. Once inside the cell, (anti)folates undergo polyglutamation in the cytosol and mitochondria, whereas the counteracting process of hydrolysis occurs in the lysosome. **FIG. 1** is adapted from Gonen et al., Drug Resistance Updates 15:183-210 (2012).

FIG. 2 depicts cellular folate metabolism and its compartmentalization in the cytosol and mitochondria. **FIG. 2** is adapted from Gonen et al., Drug Resistance Updates 15:183-210 (2012).

FIGS. 3A and 3B depict the disruption of cell polarity and tissue disorganization that is a hallmark of advanced epithelial tumors. As depicted in **FIG. 3A**, normal simple epithelium comprises a monolayer of individual cells that display a distinct apical-basal polarity. Cells are tightly packed and connected to each other by the apical junctional complexes, which separate apical and basolateral membrane domains. In normal tissue where polarity is preserved, FR- α is attached at the apical surface of cells situated away from, and out of direct contact with folates in the blood circulation. As depicted in **FIG. 3B**, cells in high-grade epithelial tumors display loss of apical-basal polarity and overall tissue disorganization, putting FR- α in direct contact with folates in blood circulation.

FIG. 4 shows molecular mechanisms underlying antifolate resistance in cancer. **FIG. 4** is adapted from Gonen et al., Drug Resistance Updates 15:183-210 (2012).

FIG. 5 shows chemical formula of L-gamma hexaglutamate pemetrexed.

FIG. 6 shows chemical formulae of example L-gamma polyglutamated antifolate compositions encompassed by the disclosure.

FIG. 7 depicts the dose response relationship of free pemetrexed L-gamma hexaglutamate (gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), pemetrexed, and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6-FR1Ab), in the NCI H2342 non-small cell lung cancer (NSCLC), adenocarcinoma subtype. The output is cell viability as measured by luciferase luminescence. As shown collectively in **FIG. 7**, the free pemetrexed gG6 appears to be the least potent in inhibited cell viability as measured by IC₅₀. Both the liposomal pemetrexed gG6 and the liposomal pemetrexed gG6-FR1Ab are 7-fold and 40-fold more potent, respectively, than free pemetrexed.

FIG. 8 presents the dose response relationship of free pemetrexed L-gamma hexaglutamate (gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), pemetrexed, and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6-FR1Ab) in the NCI H2342 non-small cell lung cancer (NSCLC), adenocarcinoma subtype depicted as the percentage of viable cells after 48 hours of treatment.

FIG. 9 presents an example dose response relationship of free pemetrexed L-gamma hexaglutamate (gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), pemetrexed, and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6-FR1Ab) in the NCI H2342 non-small cell lung cancer (NSCLC), adenocarcinoma subtype depicted as the percentage of viable cells after 48 hours of treatment.

FIG. 10 presents the dose response relationship of free pemetrexed L-gamma hexaglutamate (gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), pemetrexed, and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6-FR1Ab) in the HT-29 (colon cancer) at 48 hours. The free pemetrexed gG6 appears to be the least potent. Additionally, the liposomal pemetrexed gG6 is twice as potent as pemetrexed and the liposomal pemetrexed gG6-FR1Ab is 5-fold more potent than free pemetrexed.

FIG. 11 presents an example dose response relationship of free pemetrexed L-gamma hexaglutamate (gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), pemetrexed, and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6-FR1Ab) in the HT-29 (colon cancer) at 48 hours.

FIG. 12 shows the effect of pemetrexed, free pemetrexed L-gamma hexaglutamate (hexa gG6), liposomal pemetrexed L-gamma hexaglutamate (liposomal hexa gG6), and free pemetrexed, on the growth of ovarian cancer OAW28 cells following exposure of 256 nM of the corresponding agent for 48 hours. Liposomal pemetrexed hexa gG6 is able to enter cells more efficiently than pemetrexed hexa gG6 and pemetrexed to inhibit growth of the ovarian cancer OAW28 cells.

FIG. 13 shows the effect of free pemetrexed L-gamma hexaglutamate (hexa gG6) and liposomal pemetrexed L-gamma hexaglutamate (liposomal hexa gG6), on the growth of colon cancer SW260 cells following exposure of 256 nM of the corresponding agent for 48 hours. The untargeted and targeted liposomal pemetrexed hexa gG6 are able to enter cells more efficiently than free pemetrexed hexa gG6 to inhibit growth of the colon cancer SW260 cells.

FIG. 14 presents the relative potency of liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6) relative to pemetrexed following exposure of the cancer cell lines SW620 (CRC), HT-29 (colon cancer), H1806 (triple negative

breast cancer), OAW28 (ovarian cancer), H292 (NSCLC, adenocarcinoma subtype), and H2342 (NSCLC, adenocarcinoma subtype), over 48 hours.

FIG. 15 presents the treatment effect on HCC1806 triple negative breast cancer cells following exposure of liposomal pemetrexed L-gamma hexaglutamate (Lps hexa gG6) as compared to pemetrexed over 48 hours.

FIG. 16 presents the treatment effect on H292 non-small cell lung cancer cells following exposure of liposomal pemetrexed L-gamma hexaglutamate (Lps hexa gG6) as compared to pemetrexed over 48 hours.

FIG. 17 presents the treatment effect on OAW28 ovarian cancer cells following exposure of liposomal pemetrexed L-gamma hexaglutamate (Lps hexa gG6) as compared to pemetrexed over 48 hours.

FIG. 18 presents the treatment effect on H292 non-small cell lung cancer cells following exposure of various dose levels ranging from 16 to 128 nM of liposomal pemetrexed L-gamma hexaglutamate (Liposomal gG6), and pemetrexed over 48 hours. At each of the tested dose ranges, the liposomal pemetrexed gG6 formulation is superior to inhibiting H292 non-small cell lung cancer cells compared to pemetrexed.

FIG. 19 presents the treatment effect on HCC1806 triple negative breast cancer cells following exposure of 16 nM, 32 nM, 64 nM, and 128 nM of liposomal pemetrexed L-gamma hexaglutamate (Liposomal gG6) and pemetrexed over 48 hours. At each of the tested doses, the liposomal pemetrexed gG6 formulation is superior to pemetrexed in inhibiting HCC1806 triple negative breast cancer cells.

FIG. 20 presents the treatment effect on OAW28 ovarian cancer cells of liposomal pemetrexed L-gamma hexaglutamate (Liposomal gG6) and pemetrexed following exposure over 48 hours and over a range of concentrations. At the dose of 128 nM, pemetrexed appears to more efficiently inhibit cell proliferation than the liposomal pemetrexed gG6 liposomal formulation, whereas the liposomal formulation at the dose of 32 nM and 64 nM has a better treatment effect than pemetrexed; at 16 nM the Liposomal pemetrexed gG6 treatment effect is similar to pemetrexed.

FIG. 21 shows the toxicity of liposomal pemetrexed L-gamma hexaglutamate (Liposomal gG6) and pemetrexed on differentiating human neutrophils at 16 nM, 32 nM, 64 nM, and 128 nM. The figure demonstrates that liposomal pemetrexed gG6 is significantly less toxic to differentiating human neutrophils than pemetrexed.

FIG. 22 shows the effect of liposomal pemetrexed L-gamma hexaglutamate (liposomal gG6), and pemetrexed on AML12 liver cells following exposure over 48 hours at 16 nM, 32 nM, and 64 nM, and 128 nM of the corresponding agent. Strikingly, there does not appear to be any toxicity to the AML12 liver cells following treatment with a liposomal pemetrexed gG6 at any of the dose levels tested. In contrast, pemetrexed treatment results in a reduction in the AML12 liver cell counts of approximately 40% at all doses studied.

FIG. 23 shows the effect of liposomal L-gamma hexaglutamate (liposomal gG6) and and pemetrexed on CCD841 colon epithelium cells following exposure over 48 hours at 16 nM, 32 nM, and 64 nM, and 128 nM, of the corresponding agent. At all of the concentrations tested, pemetrexed leads to approximately a $\geq 50\%$ decrease in the number of CCD841 colon epithelium cells compared to approximately a 20% or less decrease in cell number after treatment with liposomal gG6.

DETAILED DESCRIPTION

[0034] The present application describes advances over prior cancer treatments and methods of delivering antifolates to cancer cells. In particular, the disclosure relates to a class of previously unappreciated antifolates. The invention is defined by the claims.

[0035] More specifically, the present application describes encapsulated polyglutamated antifolates such as, but not limited to, pemetrexed polyglutamate and Lometrexol polyglutamate. In a non-limiting example, the encapsulated polyglutamated antifolate (e.g., pemetrexed polyglutamate) is targeted using a targeting moiety with a specific affinity for one or more folate receptors alpha, beta and delta. In another non-limiting example, the encapsulated polyglutamated antifolate is untargeted.

[0036] Folate is a water-soluble B vitamin. The primary role of folate in the body/cell is as a cofactor to various methyltransferases involved in serine, methionine, thymidine and purine biosynthesis that are critical processes involved in cell division. The major function of folate is to mediate the transfer of one-carbon unit involved in nucleotide biosynthesis, the remethylation of homocysteine (Hcy), and biological methylation reactions. As an essential co-factor of the *de novo* biosynthesis of nucleotide, folate plays an important role in DNA synthesis, stability and integrity, and repair. Folate also provides the primary methyl group donor for the methylation of DNA (regulates gene expression), proteins (important post-translational modifications), and lipids (important in their synthesis, for example phosphatidylcholine). As explained in greater detail below, because of this, antifolates were developed as a treatment for cancer with intent to block the actions of folate, thereby inhibiting cell division, DNA/RNA synthesis and repair as well as protein synthesis.

[0037] In nature, folate occurs in animal products and in leafy vegetables in polyglutamated forms. Polyglutamates are not amenable to physiological transport. Natural polyglutamates are broken down in the jejunum primarily into monoglutamates before they are physiologically transported and taken up by the cell. Dietary folate in the form of the polyglutamates is cleaved to the monoglutamate in the jejunum where it is absorbed. Once absorbed by enterocytes,

folate is either polyglutamated and retained within the cells or released into portal circulation for various compartments for metabolism, storage, or enterohepatic recirculation. Folates enter plasma and are rapidly cleared by entering hepatocytes and other cells.

[0038] The liver takes up the majority of folate that enters portal circulation, while the remaining folate passes through the liver, enters the general circulation and is taken up by other tissues where it is converted into its polyglutamate form for intracellular storage. The liver is the primary reservoir of folates. Surgical biliary drainage can result in a reduction in serum folate within six hours, whereas dietary restriction does not produce a comparable fall for three weeks, presumably because total body stores of folate are estimated to be between 500 to 20,000 mcg. This observation indicates that there is a large enterohepatic circulation of folate.

[0039] Monoglutamates are the only circulating forms of folates in the blood and the only form of folate that is transported across the cell membrane. Once absorbed by enterocytes, folate is either polyglutamated and retained within the cells or released into portal circulation for various compartments for metabolism, storage, or enterohepatic recirculation. Once monoglutamates are taken up into cells, intracellular folates exist primarily as polyglutamates, a form that is biologically active as a cofactor to various methyltransferases involved in serine, methionine, thymidine and purine biosynthesis that are critical processes involved in cell division.

[0040] Polyglutamation involves the addition of glutamate groups in gamma linkage to the end carboxyl group of the neighboring folyl glutamate by the enzyme folylpolyglutamate synthetase (FPGS) using ATP as its energy source.

[0041] Polyglutamation refers to the addition of glutamic acid residue(s) to a molecule (such as an antifolate) so that the resultant molecule after the polyglutamation has more than one glutamic acid residue. The literature has used multiple names to refer to the glutamic acid residue such as glutamate, glutamyl group, glutamyl radical, and the like. Each of the glutamic acid residues (glutamyl groups) may independently be in the L-form or the D-form.

[0042] For example, pemetrexed, whose chemical name is "N-[4-2-(2-Amino-4,7-dihydro-4-oxo-1H -pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid" already has one glutamyl group (monoglutamated). The addition of glutamic acid residue(s) to pemetrexed as described herein results in polyglutamated pemetrexed. For example, the addition of 5 more glutamic acid residues to pemetrexed would lead to a total of 6 glutamyl groups (one from pemetrexed and 5 additional glutamyl groups added) and is referred to in this document as pemetrexed hexaglutamate or hexaglutamated pemetrexed. In the literature this entity has also be referred to as pentaglutamate pemetrexed or pemetrexed pentaglutamate.

[0043] Gamma polyglutamation refers to the addition of glutamic acid residue(s) to a molecule as described above, where the peptide bonds are between the amino group of glutamic acid and the carboxyl group at gamma carbon of the glutamic acid side chain. Alpha polyglutamation refers to the addition of glutamic acid residue(s) to a molecule, as described above, where the peptide bonds are between the amino group of glutamic acid and the carboxyl group at alpha carbon of the glutamic acid side chain.

[0044] A single molecule may be formed by gamma polyglutamation only, alpha polyglutamation only, or a combination of gamma polyglutamation and alpha polyglutamation.

[0045] Antifolates were developed more than 70 years ago as "folic acid mimic molecule" cytotoxic agents. The rationale was to design a class of molecules that would counter the action of folic acid in fast replicating cells such as cancer cells, taking advantage of physiological folate transport mechanism and their facilitative intracellular mode of action for DNA replication during the cell division. Specifically, antifolates were designed to mimic folic acid in its systemic transport, physiologic cell uptake (e.g., via reduced folate carriers (RFCs) and proton-coupled folate transporters (PCFTs)) and intracellular processing. Antifolates act specifically during DNA and RNA synthesis, exerting a cytotoxic effect during the S-phase of the cell cycle. As a result, they have a greater toxic effect on rapidly dividing cells such as malignant and myeloid cells.

[0046] Generally speaking, there are four transporters of folates and antifolates in the human body: reduced folate carriers (RFCs), folate receptors (FRs), proton-coupled folate transporters (PCFTs) and ATP-binding-cassette transporters. As noted above, none of these transport mechanisms are effective for transporting free polyglutamates across cell membranes.

[0047] RFCs are saturable anion dependent cell membrane carriers with a greater affinity for reduced folates and hydrophilic antifolates such as MTX or PMX and affinity for PMX that is 2-fold higher than that for MTX. They are members of the superfamily of solute carriers, and utilize the high transmembrane anion gradient, particularly the organic phosphate gradient which is modulated by energy status of the cell in order to achieve uphill folate transport into the cell.

[0048] The transport of cytotoxic antifolates by RFCs is not tumor specific. Because RFCs are ubiquitously expressed in normal tissues and exhibit a high level of activity at neutral pHs that are characteristic of most normal tissues, antifolates transported via RFCs have been shown to cause toxicities to a select type of normal tissues.

[0049] Folate receptors alpha, beta and delta (FR- α , FR- β and FR- δ) are high-affinity folate-binding proteins that are anchored in the cell membrane by the glycosylphosphatidylinositol (GPI) anchor domain. FRs have an especially high affinity for FA. They transport folates into cells via an endocytosis mechanism. Once in the cytoplasm, when the vesicle acidifies to a pH of 6.0-6.5, folate is released from the receptor and is exported from the endosome by a mechanism that

has been proposed to be mediated in part by PCFT. Even though FR- α has a higher affinity for its preferred substrates than RFC, its folate transport into the cell requires a series of steps such as binding, invagination, vesicle formation and translocation, acidification, and export of substrate from the vesicle into cytoplasm. Because of these multiple steps required for transport into the cell, the rate of FR-mediated folate transport is 1% of the rate mediated by RFC.

[0050] The proton-coupled folate transporter (PCFT) is a folate-H⁺symporter that functions most efficiently in an acidic extracellular environment that is, among others, characteristic of hypoxic environment. The important difference between RFC and PCFT is the optimum pH that impacts their affinities for transport substrates. At pH 7.4, the transport of antifolates is mediated predominantly by RFC as RFC activity is optimal and the activity of PCFT is minimized. Alternatively, PCFT activity is more prominent as pH is decreased. Besides mediating intestinal folate absorption and uptake into tissues in which there is an acidic extracellular environment such as solid tumors or, less efficiently, transport into tissues in a neutral pH environment, PCFT may play a role, in folate receptor-mediated endocytosis.

[0051] Several of the ATP-Binding-Cassette Transporters are low-affinity, high capacity ATP-dependent efflux pumps of folates and antifolates. These include the multidrug-resistance-associated proteins, MRP1-MRP5 and the breast-cancer resistance protein, BCRP. All of these drug resistance efflux pumps are not just involved in pumping antifolates out of the cells, but have also been shown to pump out of cells other classes of drugs.

[0052] Intracellular folate is converted to polyglutamates by FPGS, whereas GGH removes the terminal glutamates, thereby facilitating the export of folate out of the cell back to extra-cellular circulation by the ATP-Binding-Cassette Transporters, sometimes also referred to as folate efflux pumps. Without wishing to be bound by theory, polyglutamated folates are thought to be better retained in cells because they are poor substrates of the ATP-binding cassette transporters. In addition, without wishing to be bound by theory, polyglutamated folates are believed to be better substrates for intracellular folate dependent enzymes compared to monoglutamates.

[0053] As is the case for folates, antifolates such as MTX, PMX and RTX are believed to be retained in the tumor and normal cells by FPGS-induced polyglutamation and are exported from cells, after hydrolysis to monoglutamates, by GGH. As with the physiologic folate, polyglutamated antifolates are retained in cells longer, thereby increasing their cytotoxicity by extending the length of exposure. Polyglutamated antifolates generally have a higher affinity for and as a result inhibit their target folate-dependent enzymes in thymidylate and purine biosynthesis to a greater extent than monoglutamate forms. It is because of this that FPGS and GGH are considered to be important enzymes for the maintenance of intracellular homeostasis of folates and antifolates for optimal folate-dependent one-carbon transfer reactions and antifolate-induced cytotoxic effects.

[0054] Once inside the cell, folates and antifolates (such as, for example, and without limitation, pemetrexed and pralatrexate) are polyglutamated by FPGS into polyglutamates. This process is required for biological activity. For example, polyglutamation facilitates retention (increases intracellular concentrations) and increases affinity for folate-dependent enzymes (including thymidylate synthase). The ability of cells to form polyglutamates also enhances the cytotoxic action of an antifolate, thereby making antifolates very effective cytotoxic agents.

[0055] The present applicant undertook to study the cytotoxic effects of polyglutamated forms of antifolates to assess their effectiveness in treating cancerous cells. Because polyglutamated forms of antifolates are not taken up by either normal or cancer cells (as discussed in greater detail below), the applicant postulated that if such polyglutamated forms could be directly delivered to cancer cells, the toxicity of the antifolates could be improved, with reduced undesirable toxicities to normal cells that may occur as a result of efflux of excess antifolate in the cancer cells.

[0056] In particular, excess amounts of antifolate present in the cancer cells may be returned to the circulatory system via high capacity ATP-dependent efflux pumps of folates and antifolates. This is true even when the antifolates are delivered using the targeted liposomal approach described in WO 2016/25882. In other words, even though the techniques of WO 2016/25882 can deliver antifolates to cancer cells while substantially bypassing normal cells, the efflux of excess of the delivered antifolate can be returned to the circulatory system and be taken up by normal cells, resulting in undesirable toxicities. While these toxicities are significantly reduced as compared to toxicities associated with the traditional administration of free antifolates in the circulatory system of a patient, they are nonetheless undesirable. The antifolates and delivery systems described herein can further reduce the albeit significantly reduced toxic effects on normal cells achieved by WO 2016/25882, and provide even greater reductions in toxicity for normal cells.

[0057] The present inventors realized potential advantages of using polyglutamated antifolates for cancer treatment. For example, polyglutamates, especially pentaglutamates, can permit a cell to more efficiently retain its folate pools. However, due to their high negative charge and other properties, pentaglutamates currently have no known cross cell membrane transport substrates.

[0058] The present disclosure describes advanced and improved techniques to deliver to cancer cells a different class of antifolates (e.g., polyglutamates) which were previously not able to be taken up by cancer cells. Because of the lack of cross cell membrane transport, the polyglutamates delivered to the cancer cells are effectively retained within the cancer cells. This reduces and/or eliminates toxicities associated with, for example, non-polyglutamated antifolates, such as monoglutamated antifolates because the polyglutamates are not returned to the circulatory system. Indeed, even if they were, they would not be picked up by healthy cells.

[0059] The therapeutic benefits provided by polyglutamated antifolate compositions and their uses are both numerous and surprising.

[0060] First, the active form of many cytotoxic agents, and antifolates in particular, is in the polyglutamated form and therefore, the delivery of the previously undeliverable polyglutamated form of the antifolate means that the drug is immediately active. Moreover, it is known that some solid tumors are low or deficient in FPGS activity and polyglutamation level. This means that tumors that are low in FPGS activity are more resistant to chemotherapy via non-polyglutamated drugs because the drugs, after entry into the cells, are still dependent on the low endogenous FPGS activity for activation.

[0061] Second, retention of drugs in their non-polyglutamated states is also problematic. Part of the problem in treating tumors, and especially tumors with low endogenous FPGS activities, is two-fold at least. The previous paragraph discusses the issue with low activation of drugs due to low levels of FPGS. In addition to this defect, non-polyglutamated drugs such as, non-polyglutamated pemetrexed, have limited retention in cells because they can be actively transported out of the cell. The transport of non-polyglutamated drugs (e.g., non-polyglutamated pemetrexed) is in itself a two-fold problem. Firstly, if the non-polyglutamated drug is transported out of the cell, it can no longer act within the cancer cell for treatment. Also, cytotoxic agents such as antifolates that are located outside of the cell can re-enter other cells, including normal cells, and cause undesirable toxicity to normal cells. It is possible that upon entry into the cell, certain cellular processes might function to reduce the polyglutamated antifolates provided herein to monoglutamates. However, current testing suggests that the toxicity and activity of the provided polyglutamated antifolates kill the cells before such reduction occurs.

[0062] Third, because a polyglutamated antifolate is retained inside the cell and not transported extracellularly, the administration of the polyglutamated antifolate drug (e.g., intravenous administration) may be performed only for a limited time, therefore further reducing potential undesirable toxicity to normal tissue. A reduced administration time also improves patient comfort and reduces cost since the length of administration in a clinical setting can be proportionally reduced. The retention of the polyglutamated antifolate within for example, tumor cells means that the tumor cells would be less likely to recover from the treatment, even after the drug is removed from the extracellular milieu (e.g., intravenous administration is stopped).

[0063] Fourth, the activity of the disclosed liposomal polyglutamated antifolates such as the targeted- and non-targeted-pegylated liposomal polyglutamated antifolates is undiminished in tumors even if the tumor is FPGS deficient. That is because there is no reliance of FPGS to activate the drug.

[0064] For all the above stated reasons, the liposomal polyglutamated antifolate has a higher therapeutic index than a non polyglutamated antifolate that is delivered in the same method. That is, the inventors expect the drug to have minimal/reduced toxicity and for patients to tolerate higher dosages of the drug with lower side effects. The benefits described above are absent in an administered drug that is not polyglutamated.

[0065] A non-exhaustive list of polyglutamated antifolates that are encompassed by the compositions and methods of this disclosure include polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887.

[0066] In some embodiments, the pentaglutamated antifolate is a member selected from the group consisting of: pentaglutamated methotrexate (MTX), pentaglutamated pemetrexed (PMX), pentaglutamated lometrexol (LTX), pentaglutamated AG2034, pentaglutamated raltitrexed (RTX), pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887.

[0067] In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a provided liposomal composition is pentaglutamated. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a provided composition is pentaglutamated. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a liposomal composition is pentaglutamated. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a provided composition is hexaglutamated. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a liposomal composition is hexaglutamated. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in the composition has 5-10 or ≥ 4 glutamyl groups. In some embodiments, greater than 70%, 80% or 90% of the the polyglutamated antifolate in a liposomal composition has 5-10 or ≥ 4 glutamyl groups.

[0068] .In some embodiments, the alpha (L-alpha or D-alpha) or D-gamma polyglutamated antifolate compositions (e.g., polyglutamates and delivery vehicles such as liposomes containing the polyglutamates) are in an aqueous solution. In some embodiments, the polyglutamated antifolate is at a concentration of 100ng/ml to 700 mg/ml. In some embodiments, the polyglutamated antifolate is at a concentration of greater than 100ng/ml, 250 ng/ml, 500 ng/ml, 750 ng/ml, 1 ug/ml, 100 ug/ml, 250 ug/ml, 500 ug/ml, 750 ug/ml, 1 mg/ml, 100 mg/ml, 250 mg/ml, or 500 mg/ml. In some embodiments, the concentration of the liposomes containing the polyglutamated antifolates is at a concentration of 100ng/ml to 1 mg/ml. In some embodiments, the concentration of greater than 100ng/ml, 250 ng/ml, 500 ng/ml, 750 ng/ml, 1 ug/ml, 100 ug/ml, 250 ug/ml, 500 ug/ml, 750 ug/ml, or 1 mg/ml.

[0069] In some embodiments, the hexaglutamated antifolate is a member selected from the group consisting of: hexaglutamated methotrexate (MTX), hexaglutamated pemetrexed (PMX), hexaglutamated lometrexol (LTX), hexaglutamated AG2034, hexaglutamated raltitrexed (RTX), hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887.

[0070] Pemetrexed, for example, is a multitargeted antifolate that inhibits at least three enzymes involved in folate metabolism and purine and pyrimidine synthesis. These enzymes are thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycylamide ribonucleotide formyltransferase (GARFT)(see, **FIG. 2**). Pemetrexed is approved by the FDA and currently remains an important treatment for mesothelioma and non-small-cell lung cancers.

[0071] In the treatment of mesothelioma and non-small cell lung cancer, pemetrexed is approved in combination with cisplatin for the therapy of malignant mesothelioma, and for the initial as well as maintenance treatment of patients with non-small-cell lung cancers. Pemetrexed has demonstrated equivalent efficacy to docetaxel, but with significantly less toxicity, in second-line treatment of non-small-cell lung cancer. The most common and serious toxicities of pemetrexed-myelosuppression and mucositis-have been significantly ameliorated by folic acid and vitamin B12 supplementation, but undesirable toxicities remain. In some particular embodiments, the polyglutamated antifolate is polyglutamated pemetrexed (PMX). In further embodiments, the polyglutamated antifolate is pentaglutamated PMX. In further embodiments, the polyglutamated antifolate is hexaglutamated PMX.

[0072] These toxicities can be reduced, minimized and/or eliminated using a polyglutamated antifolate as described herein. In some particular embodiments described herein, the polyglutamated antifolate is polyglutamated pemetrexed (PMX). In further embodiments, the polyglutamated antifolate is pentaglutamated PMX. In further embodiments, the polyglutamated antifolate is hexaglutamated PMX.

[0073] In some particular embodiments, the polyglutamated antifolate is polyglutamated raltitrexed (RTX). In further embodiments, the polyglutamated antifolate is pentaglutamated RTX. In further embodiments, the polyglutamated antifolate is hexaglutamated RTX.

[0074] Lometrexol was the first potent antifolate inhibitory to purine synthesis due to its direct suppression of glycylamide ribonucleotide transferase (GARFT) activity. In particular embodiments, the polyglutamated antifolate is polyglutamated lometrexol (LTX). In further embodiments, the polyglutamated antifolate is pentaglutamated LTX. In further embodiments, the polyglutamated antifolate is hexaglutamated LTX

[0075] As discussed herein, the use of antifolates such as pemetrexed in clinical setting has led to a serious clinical dilemma. Fast renewing normal tissue cells such as bone marrow, GI tract and oral mucosa cells have been shown to be susceptible to the cytotoxic effect of this class of drugs, because they also replicate their DNA more frequently. Consequently, in clinical practice, use of antifolates has often resulted in severe and life-threatening hematologic and non-hematologic toxic side-effects. Furthermore, the toxicities were deemed on one hand to be dose-limiting and on the other hand to hamper the ability to achieve good efficacy. Due to their associated severe life-threatening toxicities, promising antifolates either failed during development e.g. lometrexol or have had limited use in clinical practice e.g. raltitrexed (TOMUDEX®) and pemetrexed (ALIMTA®).

[0076] The factors described herein are also at the core of the mechanisms of resistance to antifolates in general and those whose antitumor activity is potentiated by polyglutamation such as methotrexate, pemetrexed, lometrexol, and raltitrexed.

[0077] The disclosed polyglutamated antifolates provide a strategy for overcoming the pharmacological challenges associated with the dose limiting toxicities of antifolate drugs. The provided methods deliver to tumor cells a previously undeliverable (due to lack of transport mechanisms) polyglutamate form of the antifolate payload while (1) minimizing/reducing exposure to normal tissue cells, (2) optimizing/improving the cytotoxic effect of the antifolate on cancer cells and (3) minimizing/reducing the impact of the efflux pumps.

[0078] The polyglutamated chemical entities provided herein achieve many advantages over counterpart antifolates that are not delivered to cells in a polyglutamated form. For example, the polyglutamated antifolates are administered as a bolus that bypass the FPGS-induced polyglutamation activity required for antifolates such as PMX, MTX, RTX, and LTX to have increased activity (*via* increased binding affinity for folate-dependent enzymes) and cellular retention. As reflected in the figures, disclosure, and examples, herein, retention of higher polyglutamates (*e.g.*, drugs containing 4, 5, or more than 5 glutamate groups) in a cell is the direct function of their chain length. Thus, significant fractions of unbound MTX-Glu4 and most of MTX-Glu5 remain in the cells for at least 24 hours after removal of extracellular drug and continue to exert an inhibitory effect on DHFR, DNA synthesis, and cell viability.

[0079] The new chemical entities can be designed not only to increase and/or maximize tumor intracellular concentration of polyglutamated form of antifolates by carefully leveraging cell and molecular biology of the folate pathways. Certain targeted forms of new chemical entity can exploit the differential cell polarity between tumor and normal tissue, where a cancer specific morphology has been unappreciated as useful to the goal to mitigating the clinical and pharmacological challenges associated with the use of antifolates for cancer treatment. Furthermore, the new chemical entities will minimize tumor cell resistance treatment that is mediated by cell efflux pumps.

[0080] By way of example and without limitation, a targeted liposomal polyglutamated antifolate composition may

include a pegylated liposome including an entrapped and/or encapsulated polyglutamated antifolate; and a targeting moiety comprising an amino acid chain (e.g., an antibody or antibody fragment), the amino acid chain comprising a plurality of amino acids, the targeting moiety having a specific affinity for at least one type of folate receptor, the targeting moiety attached to one or both of a PEG and an exterior of the liposome. In an example embodiment, the liposome-encapsulated polyglutamated antifolate (LPA) may be a pentaglutamate form of pemetrexed or pentaglutamate form of any suitable antifolate. In an example embodiment, the liposome-encapsulated polyglutamated antifolate (LPA) may be a hexaglutamate form of pemetrexed or hexaglutamate form of any suitable antifolate.

[0081] By way of example and without limitation, an untargeted liposomal polyglutamated antifolate composition may include a pegylated liposome including an entrapped and/or encapsulated polyglutamated antifolate. In an example embodiment, the liposome-encapsulated polyglutamated antifolate (LPA) may be a pentaglutamate form of pemetrexed or pentaglutamate form of any suitable antifolate. In an example embodiment, the liposome-encapsulated polyglutamated antifolate (LPA) may be a hexaglutamate form of pemetrexed or hexaglutamate form of any suitable antifolate.

[0082] In some embodiments, a targeted, pegylated, liposomal polyglutamated antifolate composition includes a pegylated liposome including an entrapped and/or encapsulated polyglutamated antifolate; and a targeting moiety comprising an amino acid chain (e.g., an antibody or antibody fragment), the amino acid chain comprising a plurality of amino acids, the targeting moiety having a specific affinity for at least one type of folate receptor. The specific affinity may be defined, for example, and without limitation, to include an equilibrium dissociation constant (Kd) in a range of 0.5×10^{-10} to 10×10^{-6} moles [0.05 nanoMole to 10 μ Mole] for at least one type of folate receptor, the targeting moiety attached to one or both of a PEG and an exterior of the liposome.

[0083] In some embodiments, a targeted, pegylated, liposomal composition comprises a pegylated liposome including an entrapped and/or encapsulated polyglutamated antifolate; and a targeting moiety comprising an amino acid chain (e.g., an antibody or antibody fragment), the amino acid chain comprising a plurality of amino acids, the targeting moiety having a specific affinity for at least one type of folate receptor. In some embodiments, the specific affinity of the targeting moiety is an equilibrium dissociation constant (Kd) in a range of 0.5×10^{-10} to 10×10^{-6} moles for at least one type of folate receptor. In further embodiments, the targeting moiety is attached to one or both of a PEG and an exterior of the liposome.

[0084] There are at least three primary limitations in the use of the free cytotoxic agents such as antifolates that require polyglutamation for enhanced activity. The new chemical entities contemplated and disclosed herein are designed to address these fundamental limitations. These limitations include the following. The first limitation is toxicity to normal tissue cells; this toxicity is due to cell uptake of the monoglutamate form, via RFCs or PCFTs for example, and its subsequent intracellular conversion by FPGS into the polyglutamated forms with enhanced cellular cytotoxicity coupled with prolonged cell retention in normal tissue cells. The second limitation is delivering adequate amount of the most active polyglutamate form of the drug because such compound cannot cross the cell membrane. Instead, its intracellular availability depends on the ability of the cell to polyglutamate the monoglutamate form of the drug transported from extracellular environment. The third limitation is retaining within the tumor cells adequate levels of the most cytotoxic forms such as the pentaglutamate and hexaglutamated forms of the antifolate. This is due to the cancer cells ability to downregulate FPGS leading to decreased polyglutamation and, in some instances, to increase the breakdown of polyglutamated forms by GGH as well as upregulation of efflux pumps (ATP-cassettes) which have the ability to pump out of the cell mono and other lower glutamated forms (1-, 2-, 3)-glutamates, but not the higher (e.g., pentaglutamated and hexaglutamate) forms of folates and their analogs.

[0085] The polyglutamated antifolate new chemical entities contemplated and disclosed herein do not suffer from the above drawbacks and limitations, and are designed to address these and other limitations. Indeed, the new chemical entities will reduce/minimize the pharmacological challenges associated with systemic transport, cell uptake and intra tumor cell accumulation of the active form of the drug. The new chemical entities of the present disclosure preferentially target tumor cells for exposure to pentaglutamated and hexaglutamated forms of antifolate and/or minimizes exposure of such antifolate drug cocktail to normal cells, especially the high turnover (e.g., fast replicating) normal cells in the bone marrow and in the epithelial lining of the gastrointestinal tract.

[0086] In some embodiments, the disclosure provides a composition comprising a polyglutamated antifolate. In further embodiments the composition comprises pentaglutamated antifolate. In further embodiments the composition comprises hexaglutamated antifolate. According to some embodiments, the polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the polyglutamated member is pentaglutamated. In further embodiments, the polyglutamated member is hexaglutamated.

[0087] In one embodiment, the composition comprises polyglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the composition comprises pentaglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the composition comprises hexaglutamated PMX, MTX, RTX, and/or LTX.

[0088] In one embodiment, the composition comprises polyglutamated PMX. In a further embodiment the composition

comprises pentaglutamated PMX. In a further embodiment the composition comprises hexaglutamated PMX.

[0089] In another embodiment, the composition comprises polyglutamated MTX. In a further embodiment the composition comprises pentaglutamated MTX. In a further embodiment the composition comprises hexaglutamated MTX.

[0090] In another embodiment, the composition comprises polyglutamated RTX. In a further embodiment the composition comprises pentaglutamated RTX. In a further embodiment the composition comprises hexaglutamated RTX.

[0091] In an additional embodiment, the composition comprises polyglutamated LTX. In a further embodiment the composition comprises pentaglutamated LTX. In a further embodiment the composition comprises hexaglutamated LTX.

[0092] In additional embodiments, the disclosure provides a liposomal polyglutamated antifolate composition comprising a polyglutamated antifolate. According to the invention, the liposome is pegylated (PLPA). In some embodiments, the PLPA composition comprises a pentaglutamated antifolate. In some embodiments, the PLPA composition comprises a hexaglutamated antifolate. In some embodiments, the PLPA liposome is anionic or neutral. In other embodiments, the PLPA liposome is cationic. In some embodiments, the PLPA composition comprises at least 10% liposome entrapped polyglutamated antifolate. In some embodiments, the PLPA liposomes have a diameter in the range of 20 nm to 200 nm. In further embodiments, the liposomes have a diameter in the range of 80 nm to 120 nm.

[0093] According to some embodiments, the LPA comprises a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In further embodiments, the polyglutamated antifolate is pentaglutamated. In further embodiments, the polyglutamated antifolate is hexaglutamated.

[0094] In one embodiment, the LPA comprises polyglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the LPA comprises pentaglutamated PMX, MTX, RTX, and/or LTX. In a further embodiment the LPA comprises hexaglutamated PMX, MTX, RTX, and/or LTX.

[0095] In one embodiment, the LPA comprises polyglutamated PMX. In a further embodiment the LPA comprises pentaglutamated PMX. In a further embodiment the LPA comprises hexaglutamated PMX.

[0096] In another embodiment, the LPA comprises polyglutamated MTX. In a further embodiment the LPA comprises pentaglutamated MTX. In a further embodiment the LPA comprises hexaglutamated MTX.

[0097] In another embodiment, the LPA comprises polyglutamated RTX. In a further embodiment the LPA comprises pentaglutamated RTX. In a further embodiment the LPA comprises hexaglutamated RTX.

[0098] In an additional embodiment, the LPA comprises polyglutamated LTX. In a further embodiment the LPA comprises pentaglutamated LTX. In a further embodiment the LPA comprises hexaglutamated LTX.

Liposome

[0099] In some embodiments, a targeted pegylated liposomal polyglutamated antifolate is provided. In some embodiments, the targeted pegylated liposomal polyglutamated antifolate may comprise a liposome including an interior space; a polyglutamated antifolate disposed within the interior space; a PEG molecule attached to an exterior of the liposome; and a targeting moiety comprising a protein (e.g., an antibody or antibody fragment) with specific affinity for at least one antigen expressed on the surface of the cancer cell (e.g., a folate receptor (e.g., FR- α , FR- β and/or FR- δ)) the targeting moiety attached to at least one of the PEG and the exterior of the liposome.

[0100] In other embodiments, an untargeted liposomal polyglutamated antifolate is provided.

[0101] The liposomes contained in the targeted and untargeted liposome composition can be any liposome known in the art. However, it will be understood by one skilled in the art that liposomal encapsulation of any particular drug, such as, and without limitation, the polyglutamated antifolates discussed herein, may involve substantial routine experimentation to achieve a useful and functional liposomal formulation. In general, the provided liposomes may have any liposome structure, e.g., structures having an inner space sequestered from the outer medium by one or more lipid bilayers, or any microcapsule that has a semi-permeable membrane with a lipophilic central part where the membrane sequesters an interior. The lipid bilayer can be any arrangement of amphiphilic molecules characterized by a hydrophilic part (hydrophilic moiety) and a hydrophobic part (hydrophobic moiety). Usually amphiphilic molecules in a bilayer are arranged into two dimensional sheets in which hydrophobic moieties are oriented inward the sheet while hydrophilic moieties are oriented outward. Amphiphilic molecules forming the provided liposomes can be any known or later discovered amphiphilic molecules, e.g., lipids of synthetic or natural origin or biocompatible lipids. The liposomes can also be formed by amphiphilic polymers and surfactants, e.g., polymerosomes and niosomes. For the purpose of this disclosure, without limitation, these liposome-forming materials also are referred to as "lipids".

[0102] The liposome composition formulations provided herein can be in liquid or dry form such as a dry powder or dry cake. The dry powder or dry cake may have undergone primary drying under, for example, lyophilization conditions or optionally, the dry cake or dry powder may have undergone both primary drying only or both primary drying and secondary drying. In the dry form, the powder or cake may, for example, have between 1% to 6% moisture, for example, such as between 2% to 5% moisture or between 2% to 4% moisture. One example method of drying is lyophilization (also called

freeze-drying, or cryodesiccation). Any of the compositions and methods of the disclosure may include liposomes, lyophilized liposomes or liposomes reconstituted from lyophilized liposomes. In some embodiments, the disclosed compositions and methods include one or more lyoprotectants or cryoprotectants. These protectants are typically polyhydroxy compounds such as sugars (mono-, di-, and polysaccharides), polyalcohols, and their derivatives, glycerol, or polyethyleneglycol, trehalose, maltose, sucrose, glucose, lactose, dextran, glycerol, or aminoglycosides. In further

embodiments, the lyoprotectants or cryoprotectants comprise up to 10% or up to 20% of a solution outside the liposome, inside the liposome, or both outside and inside the liposome.

[0103] In some embodiments, the liposomes include a steric stabilizer that increases their longevity in circulation. One or more steric stabilizers such as a hydrophilic polymer (Polyethylene glycol (PEG)), a glycolipid (monosialoganglioside (GM1)) or others occupies the space immediately adjacent to the liposome surface and excludes other macromolecules from this space. Consequently, access and binding of blood plasma opsonins to the liposome surface are hindered, and thus interactions of macrophages with such liposomes, or any other clearing mechanism, are inhibited and longevity of the liposome in circulation is enhanced. In some embodiments, the steric stabilizer or the population of steric stabilizers is a PEG or a combination comprising PEG. In further embodiments, the steric stabilizer is a PEG or a combination comprising PEG with a number average molecular weight (Mn) of 200 to 5000 daltons. These PEG(s) can be of any structure such as linear, branched, star or comb structure and are commercially available.

[0104] The diameter of the disclosed liposomes is not particularly limited. In some embodiments, the liposomes have a diameter in the range of, for example, 30-150 nm (nanometer). In other embodiments, the liposomes have a diameter in the range of 40-70 nm.

[0105] The properties of liposomes are influenced by the nature of lipids used to make the liposomes. A wide variety of lipids have been used to make liposomes. These include cationic, anionic and neutral lipids. In some embodiments, the liposomes comprising the polyglutamated antifolate are anionic or neutral. In other embodiments, the provided liposomes are cationic. The determination of the charge (e.g., anionic, neutral or cationic) can routinely be determined by measuring the zeta potential of the liposome. The zeta potential of the liposome can be positive, zero or negative. In some embodiments, the zeta potential of the liposome is less than or equal to zero. In some embodiments, the zeta potential of the liposome is in a range of 0 to -150 mV. In another embodiment, the zeta potential of the liposome is in the range of -30 to -50 mV.

[0106] In some embodiments, cationic lipids are used to make cationic liposomes which are commonly used as gene transfection agents. The positive charge on cationic liposomes enables interaction with the negative charge on cell surfaces. Following binding of the cationic liposomes to the cell, the liposome is transported inside the cell through endocytosis.

[0107] In some embodiments, a neutral to anionic liposome is used. In one embodiment, an anionic liposome is used. Using a mixture of, for example, neutral lipids such as HSPC and anionic lipids such as PEG-DSPE results in the formation of anionic liposomes which are less likely to non-specifically bind to normal cells. Specific binding to tumor cells can be achieved by using a tumor targeting antibody such as, for example, a folate receptor antibody, including, for example, folate receptor alpha antibody, folate receptor beta antibody and/or folate receptor delta antibody.

[0108] As an example, at least one (or some) of the lipids is/are amphipathic lipids, defined as having a hydrophilic and a hydrophobic portions (typically a hydrophilic head and a hydrophobic tail). The hydrophobic portion typically orients into a hydrophobic phase (e.g., within the bilayer), while the hydrophilic portion typically orients toward the aqueous phase (e.g., outside the bilayer). The hydrophilic portion can comprise polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxy and other like groups. The hydrophobic portion can comprise apolar groups that include without limitation long chain saturated and unsaturated aliphatic hydrocarbon groups and groups substituted by one or more aromatic, cyclo-aliphatic or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids and sphingolipids.

[0109] Typically, for example, the lipids are phospholipids. Phospholipids include without limitation phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and the like. It is to be understood that other lipid membrane components, such as cholesterol, sphingomyelin, and cardiolipin, can be used.

[0110] The lipids comprising the liposomes provided herein can be anionic and neutral (including zwitterionic and polar) lipids including anionic and neutral phospholipids. Neutral lipids exist in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, dioleoylphosphatidylglycerol (DOPG), diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides and diacylglycerols. Examples of zwitterionic lipids include without limitation dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), and dioleoylphosphatidylserine (DOPS). Anionic lipids are negatively charged at physiological pH. These lipids include without limitation phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecyl canoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0111] Collectively, anionic and neutral lipids are referred to herein as non-cationic lipids. Such lipids may contain

phosphorus but they are not so limited. Examples of non-cationic lipids include lecithin, lysolecithin, phosphatidylethanolamine, lysophosphatidylethanolamine, dioleoylphosphatidylethanolamine (DOPE), dipalmitoyl phosphatidylethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl 1-ethanolamine (DSPE), palmitoyloleoyl-phosphatidylethanolamine (POPE) palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), palmitoyloleoylphosphatidylglycerol (POPG), 16-0-monomethyl PE, 16-0- dimethyl PE, 18-1-trans PE, palmitoyloleoyl-phosphatidylethanolamine (POPE), 1-stearoyl-2-oleoylphosphatidylethanolamine (SOPE), phosphatidylserine, phosphatidylinositol, sphingomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, and cholesterol.

[0112] The liposomes may be assembled using any liposomal assembly method using liposomal components (also referred to as liposome components) known in the art. Liposomal components include, for example, lipids such as DSPE, HSPC, cholesterol and derivatives of these components. Other suitable lipids are commercially available for example, from Avanti Polar Lipids, Inc. (Alabaster, Alabama, U.S.A.). A partial listing of available negatively or neutrally charged lipids suitable for making anionic liposomes, can be, for example, at least one of the following: DLPC, DMPC, DPPC, DSPC, DOPC, DMPE, DPPE, DOPE, DMPA•Na, DPPA•Na, DOPA•Na, DMPG•Na, DPPG•Na, DOPG•Na, DMPS•Na, DPPS•Na, DOPS•Na, DOPE-Glutaryl•(Na)₂, Tetramyristoyl Cardiolipin•(Na)₂, DSPE-mPEG-2000•Na, DSPE-mPEG-5000•Na, and DSPE-Maleimide PEG-2000•Na.

[0113] Lipid derivatives can include, for example, at least, the bonding (preferably covalent bonding) of one or more steric stabilizers and/or functional groups to the liposomal component after which the steric stabilizers and/or functional groups should be considered part of the liposomal components. Functional groups comprise groups that can be used to attach a liposomal component to another moiety such as a protein. Such functional groups include, at least, maleimide. These steric stabilizers include at least one from the group consisting of polyethylene glycol (PEG); poly-L-lysine (PLL); monosialoganglioside (GM1); poly(vinyl pyrrolidone) (PVP); poly(acrylamide) (PAA); poly(2-methyl-2-oxazoline); poly(2-ethyl-2-oxazoline); phosphatidyl polyglycerol; poly[N-(2-hydroxypropyl) methacrylamide]; amphiphilic poly-N-vinylpyrrolidones; L-amino-acid-based polymer; and polyvinyl alcohol.

[0114] Since the components of a liposome can include any molecule(s) (*i.e.*, chemical/reagent/protein) that is bound to it, in some embodiments, the components of the provided liposomes include, at least, a member selected from the group DSPE, DSPE-PEG, DSPE-maleimide, HSPC; HSPC-PEG; HSPC-maleimide; cholesterol; cholesterol-PEG; and cholesterol-maleimide. In some embodiments, the components of the provided liposomes include DSPE, DSPE-PEG, DSPE-maleimide, HSPC; HSPC-PEG; HSPC-maleimide; cholesterol; cholesterol-PEG; and cholesterol-maleimide. In an example embodiment, the liposomal components that make up the liposome comprises DSPE; DSPE-FITC; DSPE-maleimide; cholesterol; and/or HSPC.

[0115] At least one component of the liposome lipid bilayer may be functionalized (or reactive). As used herein, a functionalized component is a component that comprises a reactive group that can be used to crosslink reagents and moieties to the lipid. If the lipid is functionalized, any liposome that it forms is also functionalized. The reactive group may be one that will react with a crosslinker (or other moiety) to form crosslinks. The reactive group in the liposome lipid bilayer is located anywhere on the lipid that allows it to contact a crosslinker and be crosslinked to another moiety (*e.g.*, a steric stabilizer or targeting moiety). In some embodiments, the reactive group is in the head group of the lipid, including for example a phospholipid. In some embodiments, the reactive group is a maleimide group. Maleimide groups can be crosslinked to each other in the presence of dithiol crosslinkers including but not limited to dithiolthrietol (DTT).

[0116] It is to be understood that the use of other functionalized lipids, other reactive groups, and other crosslinkers beyond those described above is further contemplated. In addition to the maleimide groups, other examples of contemplated reactive groups include but are not limited to other thiol reactive groups, amino groups such as primary and secondary amines, carboxyl groups, hydroxyl groups, aldehyde groups, alkyne groups, azide groups, carbonyls, halo acetyl (*e.g.*, iodoacetyl) groups, imidoester groups, N-hydroxysuccinimide esters, sulfhydryl groups, and pyridyl disulfide groups.

[0117] Functionalized and non-functionalized lipids are available from a number of commercial sources including Avanti Polar 5 Lipids (Alabaster, Ala.).

[0118] In some embodiments, the provided liposomes further comprise an immunostimulatory agent, a detectable marker, or both disposed on its exterior. The immunostimulatory agent or detectable marker can be ionically bonded or covalently bonded to an exterior of the liposome, including, for example, optionally to a steric stabilizer component of the liposome.

[0119] The terms immunostimulatory agents, also known as immunostimulants, and immunostimulators, refer to substances that stimulate the immune system by inducing activation or increasing activity of any of its components. These immunostimulatory agents can include one or more of a hapten, an adjuvant, a protein immunostimulating agent, a nucleic acid immunostimulating agent, and a chemical immunostimulating agent. Many adjuvants contain a substance designed to stimulate immune responses, such as lipid A, Bordetella pertussis or Mycobacterium tuberculosis derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete

Adjuvant (Difco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.); AS-2 (SmithKline Beecham, Philadelphia, Pa.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, can also be used as adjuvants. In an example embodiment, the immunostimulant can be at least one selected from the group consisting of fluorescein, DNP, beta glucan, beta-1,3-glucan, beta-1,6-glucan.

[0120] A detectable marker may, for example, include, at least, a radioisotope, a fluorescent compound, a bioluminescent compound, chemiluminescent compound, a metal chelator, an enzyme, a dye, an ink, a magnetic compound, a biocatalyst or a pigment that is detectable by any suitable means known in the art, e.g., magnetic resonance imaging (MRI), optical imaging, fluorescent/luminescent imaging, or nuclear imaging techniques.

[0121] In some embodiments, the immunostimulatory agent and/or detectable marker is attached to the exterior by co-incubating it with the liposome. For example, the immunostimulatory agent and/or detectable marker may be associated with the liposomal membrane by hydrophobic interactions or by an ionic bond such as an avidin/biotin bond or a metal chelation bond (e.g., Ni-NTA). Alternatively, the immunostimulatory agent or detectable marker may be covalently bonded to the exterior of the liposome such as, for example, by being covalently bonded to a liposomal component or to the steric stabilizer which is the PEG.

[0122] One example reagent is fluorescein isothiocyanate (FITC) which, based on experiments, can serve as both an immunostimulant and a detectable marker.

[0123] In further non-limiting embodiments, the provided liposomes enclose an interior space. In some embodiments, the interior space comprises, but is not limited to, an aqueous solution. In some embodiments, the interior space comprises a polyglutamated antifolate as provided herein. In some embodiments, the interior space further comprises a pharmaceutically acceptable carrier such as trehalose. In an additional embodiment, the trehalose is present at about 5% to 20% weight percent of trehalose or any combination of one or more lyoprotectants or cryoprotectants at a total concentration of 5% to 20%. In some embodiments, the interior space comprises buffer. In further embodiments, the buffer is HEPES buffer or citrate buffer. In yet further embodiments, the citrate buffer is at a concentration of between 5 to 200 mM. In some embodiments, the interior space has a pH of between 2.8 to 6. In additional embodiments, the interior space of the liposome comprises sodium acetate and/or calcium acetate. In some embodiments, the interior space of the liposome comprises a total concentration of sodium acetate and calcium acetate of between 50 mM to 500 mM.

[0124] In some embodiments, the targeted pegylated liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate comprises a medium comprising a liposome including an interior space; an aqueous polyglutamated antifolate disposed within the interior space; and a targeting moiety comprising a protein with specific affinity for at least one folate receptor, and wherein the targeting moiety disposed at the exterior of the liposome. In some embodiments, the medium is an aqueous solution. In some embodiments, the interior space, the exterior space (e.g., the medium), or both the interior space and the medium contains one or more lyoprotectants or cryoprotectants which are listed above. In some embodiments, the cryoprotectant is mannitol, trehalose, sorbitol, and/or sucrose.

[0125] In some embodiments, an untargeted liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate comprises a medium comprising a liposome including an interior space; and an aqueous polyglutamated antifolate disposed within the interior space. In some embodiments, the medium is an aqueous solution. In some embodiments, the interior space, the exterior space (e.g., the medium), or both the interior space and the medium contains one or more lyoprotectants or cryoprotectants which are listed above. In some embodiments, the cryoprotectant is mannitol, trehalose, sorbitol, and/or sucrose.

[0126] As discussed above, the liposomes may comprise a steric stabilizer that can increase their longevity in circulation. For those embodiments, which incorporate a steric stabilizer, the steric stabilizer may be at least one member selected from the group consisting of polyethylene glycol (PEG), poly-L-lysine (PLL), monosialoganglioside (GM1), poly(vinyl pyrrolidone) (PVP), poly(acrylamide) (PAA), poly(2-methyl-2-oxazoline), poly(2-ethyl-2-oxazoline), phosphatidyl polyglycerol, poly[N-(2-hydroxypropyl) methacrylamide], amphiphilic poly-N-vinylpyrrolidones, L-amino-acid-based polymer, and polyvinyl alcohol. In some embodiments, the steric stabilizer or the population of steric stabilizer is PEG. In one embodiment, the steric stabilizer is a PEG. In a further embodiment, the PEG has a number average molecular weight (Mn) of 200 to 5000 daltons. These PEG(s) can be of any structure such as linear, branched, star or comb structure and are commercially available.

[0127] In some embodiments, the liposomal polyglutamated antifolate (LPA or PLPA) is water soluble. That is, the liposomal polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate is in the form of an aqueous solution. In some embodiments, the LPA or PLPA comprises an interior space that contains less than 200,000 molecules of the polyglutamated antifolate. In some embodiments, the LPA or PLPA contains between 10,000 to 100,000 molecules of the polyglutamated antifolate. In further embodiments, the LPA or PLPA contains between 10,000 to 100,000 molecules of pentaglutamated antifolate. In further embodiments, the LPA or PLPA contains between 10,000 to 100,000 molecules of pentaglutamated antifolate.

[0128] In some embodiments, the interior space of the LPA or PLPA contains less than 200,000 molecules of a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the liposome contains less than 200,000 molecules of polyglutamated PMX. In some embodiments, the liposome contains less than 200,000 molecules of polyglutamated MTX. In some embodiments, the liposome contains less than 200,000 molecules of polyglutamated RTX. In some embodiments, the liposome contains less than 200,000 molecules of molecules of pentaglutamated LTX. In some embodiments, the liposome contains less than 200,000 molecules of molecules of hexaglutamated LTX.

[0129] In some embodiments, the interior space of the LPA or PLPA contains between 10,000 to 100,000 molecules of a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of polyglutamated PMX. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of polyglutamated MTX. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of polyglutamated RTX. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of pentaglutamated LTX. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of hexaglutamated LTX.

[0130] In some embodiments, the interior space of the LPA or PLPA contains less than 200,000 molecules of a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the liposome contains less than 200,000 molecules of pentaglutamated PMX. In some embodiments, the liposome contains less than 200,000 molecules of pentaglutamated MTX. In some embodiments, the liposome contains less than 200,000 molecules of pentaglutamated RTX. In some embodiments, the liposome contains less than 200,000 molecules of pentaglutamated RTX.

[0131] In some embodiments, the interior space of the LPA or PLPA contains less than 200,000 molecules of a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In some embodiments, the liposome contains less than 200,000 molecules of hexaglutamated PMX. In some embodiments, the liposome contains less than 200,000 molecules of hexaglutamated MTX. In some embodiments, the liposome contains less than 200,000 molecules of hexaglutamated RTX. In some embodiments, the liposome contains less than 200,000 molecules of hexaglutamated RTX.

[0132] According to the invention, the liposomal polyglutamated antifolate is pegylated (*i.e.*, a pegylated liposomal polyglutamated (*e.g.*, pentaglutamated or hexaglutamated) antifolate (PLPA or PLPNA)). In some embodiments, the PLPA or PLPNA is water soluble. That is, the PLPA or PLPNA is in the form an aqueous solution. In some embodiments, the PLPA or PLPNA comprises an interior space that contains less than 200,000 molecules of the polyglutamated antifolate. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of the polyglutamated antifolate. In further embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated antifolate. In further embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated antifolate.

[0133] In some embodiments, the interior space of the PLPA or PLPNA contains less than 200,000 molecules of a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the liposome contains less than 200,000 molecules of polyglutamated PMX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of polyglutamated MTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of polyglutamated RTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of pentaglutamated LTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of hexaglutamated LTX.

[0134] In some embodiments, the interior space of the PLPA or PLPNA contains between 10,000 to 100,000 molecules of a polyglutamated antifolate selected from the group consisting of: polyglutamated MTX, polyglutamated PMX, polyglutamated LTX, polyglutamated AG2034, polyglutamated RTX, polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the liposome contains between 10,000 to 100,000 molecules of polyglutamated PMX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of polyglutamated MTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of polyglutamated RTX. In some

embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated LTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated LTX.

[0135] In further embodiments, the interior space of the PLPA or PLPNA contains less than 200,000 molecules of a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of pentaglutamated PMX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of pentaglutamated MTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of pentaglutamated RTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of pentaglutamated RTX.

[0136] In further embodiments, the interior space of the PLPA or PLPNA contains less than 200,000 molecules of a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of hexaglutamated PMX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of hexaglutamated MTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of hexaglutamated RTX. In some embodiments, the PLPA or PLPNA contains less than 200,000 molecules of hexaglutamated RTX.

[0137] In further embodiments, the interior space of the PLPA or PLPNA contains between 10,000 to 100,000 molecules of a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated PMX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated MTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated RTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of pentaglutamated RTX.

[0138] In further embodiments, the interior space of the PLPA or PLPNA contains between 10,000 to 100,000 molecules of a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated PMX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated MTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated RTX. In some embodiments, the PLPA or PLPNA contains between 10,000 to 100,000 molecules of hexaglutamated RTX.

[0139] In some embodiments, the pH of solutions comprising the liposome composition is from pH 5 to 8 or from pH 2 to 6.

Targeted liposomes

[0140] In some embodiments, the disclosure provides a liposomal polyglutamated antifolate composition wherein the liposome is pegylated and comprises a polyglutamated antifolate and targeting moiety attached to one or both of a PEG and the exterior of the liposome, and wherein the targeting moiety has a specific affinity for a surface antigen on a target cell of interest. Such liposomes may generally be referred to herein as "targeted liposomes," e.g., liposomes including one or more targeting moieties or biodistribution modifiers on the surface of, or otherwise attached to, the liposomes. The targeting moiety of the targeted liposomes can be any moiety or agent that is capable of specifically binding a desired target (e.g., an antigen target expressed on the surface of a target cell of interest). In one embodiment, the targeted liposome specifically and preferentially binds to a target on the surface of a target cell of interest that internalizes the targeted liposome into which the liposome encapsulated polyglutamated cytotoxic agent (e.g., polyglutamated antifolates, such as pentaglutamated or hexaglutamated PMX, LTX, and MTX) exerts its cytotoxic effect. In further embodiments, the target cell is a cancer cell, a tumor cell or a metastatic cell. In some embodiments, the targeting liposomes are immunoliposomes.

[0141] The term attach or attached refers, for example, to any type of bonding such as covalent bonding, ionic bonding (e.g., avidin-biotin) bonding by hydrophobic interactions, and bonding via functional groups such as maleimide, or linkers such as PEG. For example, a detectable marker, a steric stabilizer, a liposome, a liposomal component, an immunostimulating agent may be attached to each other directly, by a maleimide functional group, or by a PEG-maleimide group.

[0142] In some embodiments, the targeting moiety attached to the liposome is a polypeptide. In further embodiments, the targeting moiety is an antibody or a fragment of an antibody. In additional embodiments, the targeting moiety comprises one or more of an antibody, a humanized antibody, an antigen binding fragment of an antibody, a single chain antibody, a

single-domain antibody, a bi-specific antibody, a synthetic antibody, a pegylated antibody, and a multimeric antibody. In additional embodiments, the targeting moiety has the specific affinity for an epitope on a tumor cell surface antigen that is present on a tumor cell but absent or inaccessible on a non-tumor cell. In some embodiments, the targeting moiety further comprises one or more of an immunostimulatory agent, a detectable marker and a maleimide disposed on at least one of the PEG and the exterior of the liposome. In some embodiments, the targeting moiety of the liposomal polyglutamated antifolate (LPA) or a pegylated liposomal polyglutamated antifolate (PLPA) liposome is anionic or neutral. In other embodiments, the targeting moiety of the LPA or PLPA liposome is cationic. In some embodiments, the targeting moiety LPA or PLPA liposome composition comprises at least 10% liposome entrapped polyglutamated antifolate. In some embodiments, the targeting moiety-LPA or targeting moiety-PLPA liposomes have a diameter in the range of 20 nm to 200 nm. In further embodiments, the liposomes have a diameter in the range of 80 nm to 120 nm.

[0143] In some embodiments, the targeting moiety-LPA or targeting moiety-PLPA comprises a polypeptide targeting moiety such as an antibody or an antibody fragment and the targeting moiety binds a target antigen with an equilibrium dissociation constant (Kd) in a range of 0.5×10^{-10} to 10×10^{-6} as determined using BIACORE analysis. In further embodiments, the targeting moiety-LPA or targeting moiety-PLPA comprises a polypeptide targeting moiety.

[0144] In some embodiments, the targeting moiety-LPA or targeting moiety-PLPA comprises a polypeptide targeting moiety such as an antibody or an antibody fragment and the targeting moiety has a specific affinity for a target antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG), SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98.

[0145] In further embodiments, the targeting moiety comprises a polypeptide targeting moiety such as an antibody or an antibody fragment and the targeting moiety has binding specificity for a folate receptor. In some embodiments the targeting moiety binds a folate receptor with an equilibrium dissociation constant (Kd) in a range of 0.5×10^{-10} to 10×10^{-6} as determined using BIACORE analysis. In some embodiments, the folate receptor bound by the targeting moiety is one or more folate receptors selected from the group consisting of: folate receptor alpha (FR- α), folate receptor beta (FR- β), and folate receptor delta (FR- δ).

[0146] In some embodiments, the targeting moiety is an antibody or antigen binding portion of an antibody that specifically binds a target of interest expressed on the surface of a targeted cell of interest. In some embodiments, the targeting moiety is a full-length antibody. In some embodiments, the targeting moiety is an antigen binding portion of an antibody. In some embodiments, the targeting moiety comprises one or more complementarity determining regions (CDRs) of antibody origin. Examples of suitable proteins that can serve as targeting moieties for the disclosed targeted liposomes include a full-length human antibody, a humanized antibody, a chimeric antibody, an antigen binding fragment of an antibody, a single chain antibody, a single-domain antibody, a bi-specific antibody, a synthetic antibody, a pegylated antibody and a multimeric antibody. The antibody of the provided targeted liposomes can have a combination of the above characteristics. For example, a humanized antibody can be an antigen binding fragment and can be pegylated and multimerized as well.

[0147] The term "humanized antibody" refers to forms of non-human (e.g., murine) antibodies that are specific immunoglobulin chains, chimeric immunoglobulins, or fragments thereof that contain minimal non-human (e.g., murine) sequences. Typically, humanized antibodies are human immunoglobulins in which residues from the complementary determining region (CDR) are replaced by residues from the CDR of a non-human species (e.g. mouse, rat, rabbit, and hamster) that have the desired specificity, affinity, and capability (Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeven et al., Science 239:1534-1536 (1988)). In some instances, the Fv framework region (FR) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, affinity, and capability. The humanized antibody can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or capability. In general, the humanized antibody will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. Nos. 5,225,539 and 5,639,641.

[0148] As discussed herein, folate receptors (FRs) are distinct from reduced folate carriers (RFCs) and exploit different pathways for bringing folates and antifolates into cells. In some embodiments, the targeting moiety specifically binds a folate receptor. In further embodiments, the targeting moiety specifically binds a folate receptor selected from folate receptor alpha, folate receptor beta and folate receptor delta. Antibodies to folate receptor alpha can routinely be generated using techniques known in the art. Moreover, the sequences of numerous anti-folate receptor antibodies are in the public domain and/or commercially available and are readily obtainable.

[0149] Murine antibodies against folate receptor are examples of antibodies that can be used as targeting moieties of the disclosed targeted liposome is a murine antibody against folate receptor. The sequence of these antibodies are known and are described, for example, in U.S. Pat. Nos. 5,646,253; 8,388,972; 8,871,206; and 9,133,275 and in Intl. Appl. Nos. PCT/US2011/056966 and PCT/US2012/046672. For example, based on the sequences disclosed already in the public domain, the gene for the antibodies can be synthesized and placed into a transient expression vector and the antibody was produced in HEK-293 transient expression system. The antibody can be a complete antibody, a Fab, or any of the various antibody variations discussed herein or otherwise known in the art.

[0150] In some embodiments, the targeted liposomes comprise from 30 to 500 targeting moieties (e.g., 30 to 250 targeting moieties or 30-200 targeting moieties). In some embodiments, the provided targeted liposomes contain less than 220 targeting moieties, less than 200 targeting moieties, or less than targeting 175 moieties. In some embodiments, the targeting moiety is non-covalently bonded to the outside of the liposome (e.g., via ionic interaction or a GPI anchor).

[0151] In some embodiments, the molecules on the outside of the targeted liposome include a lipid, a targeting moiety, a steric stabilizer (e.g., a PEG), a maleimide, and a cholesterol. In some embodiments, the targeting moiety is covalently bound *via* a maleimide functional group. In some embodiments, the targeting moiety is covalently bound to a liposomal component or a steric stabilizer such as a PEG molecule. In some embodiments, all the targeting moieties are bound to one component of the liposome such as a PEG. In other embodiments, the targeting moieties are bound to different components of the liposome. For example, some targeting moieties may be bound to the lipid components or cholesterol, some targeting moieties may be bound to the steric stabilizer (e.g., PEG) and still other targeting moieties may be bound to a detectable marker or to another targeting moiety.

[0152] In some embodiments, the targeting moiety of the targeted liposome has affinity and specificity (*i.e.*, specifically binds) for an antigen expressed on the surface of a cancer cell. In further some embodiments, the targeting moiety of the targeted liposome has affinity and specificity for one or more antigens selected from the group consisting of folate receptor alpha, folate receptor beta, and folate receptor delta. In one embodiment, the targeting moiety has specific affinity (*i.e.*, specifically binds) an antigen selected from the group consisting of folate receptor alpha, folate receptor beta, and folate receptor delta. In a further embodiment, the targeting moiety has specific affinity for at least two antigens selected from the group consisting of folate receptor alpha, folate receptor beta, and folate receptor delta. In another embodiment, the targeting moiety has specific affinity for three antigens which are, for example, folate receptor alpha; folate receptor beta; and folate receptor delta. The targeting moiety may have affinity and specificity to an epitope of the antigen because sometimes a targeting moiety does not bind the complete antigen but just an epitope of many epitopes in an antigen.

[0153] The terms "epitope" or "antigenic determinant" are used interchangeably herein and refer to that portion of an antigen capable of being recognized and specifically bound by a particular antibody or binding moiety. When the antigen is a polypeptide, epitopes can be formed both from contiguous amino acids and noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding are typically lost upon protein denaturing. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation.

[0154] In some embodiments, the targeting moiety has specific affinity for an epitope on a tumor cell surface antigen that is present on a tumor cell but absent or inaccessible on a non-tumor cell. For example, in some situations, the tumor antigen is on the surface of both normal cells and malignant cancer cells but the tumor epitope is only exposed in a cancer cell. As a further example, a tumor antigen may experience a conformation change in cancer causing cancer cell specific epitopes to be present. A targeting moiety with specific affinity to epitopes described herein are useful and are encompassed by the disclosed compositions and methods. In some embodiments, the tumor cell with the cancer cell specific epitope(s) is a cancer cell. Examples of such tumor cell surface antigens include, folate receptor alpha, folate receptor beta and folate receptor delta.

[0155] In some embodiments, the liposome composition is provided as a pharmaceutical composition containing the liposome and a carrier, e.g., a pharmaceutically acceptable carrier. Examples of pharmaceutically acceptable carriers contained in the provided pharmaceutical compositions include normal saline, isotonic dextrose, isotonic sucrose, Ringer's solution, and Hanks' solution. In some embodiments, a buffer substance is added to maintain an optimal pH for storage stability of the pharmaceutical composition. In some embodiments, the pH of the pharmaceutical composition is between 6.0 and 7.5. In some embodiments, the pH is between 6.3 and 7.0. In further embodiments, the pH is 6.5. Ideally the pH of the pharmaceutical composition allows for both stability of liposome membrane lipids and retention of the entrapped entities. Histidine, hydroxyethylpiperazine-ethylsulfonate (HEPES), morpholinoethylsulfonate (MES), succinate, tartrate, and citrate, typically at 2-20 mM concentration, are example buffer substances. Other suitable carriers include, e.g., water, buffered aqueous solution, 0.4% NaCl, and 0.3% glycine. Protein, carbohydrate, or polymeric stabilizers and tonicity adjusters can be added, e.g., gelatin, albumin, dextran, or polyvinylpyrrolidone. The tonicity of the composition can be adjusted to the physiological level of 0.25-0.35 mol/kg with glucose or a more inert compound such as lactose, sucrose, mannitol, or dextrin. These compositions can routinely be sterilized using conventional, sterilization techniques known in the art (e.g., by filtration). The resulting aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous medium prior to

administration.

[0156] The provided pharmaceutical liposome compositions can also contain other pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, and tonicity adjusting agents, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the liposome suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alpha-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

[0157] The liposome concentration in the provided fluid pharmaceutical formulations can vary widely depending upon need, e.g., from less than about 0.05% usually or at least about 2-10% to as much as 30 to 50% by weight and can be selected primarily by fluid volumes, and viscosities, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, liposome pharmaceutical compositions composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0158] Some embodiments relate to a method of delivering a targeted pegylated liposomal formulation of a polyglutamated antifolate, to a tumor expressing folate receptor on its surface. An example method comprises the step of administering at least one of any of the compositions comprising a liposome in this disclosure in an amount to deliver a therapeutically effective dose of the targeted pegylated liposomal polyglutamated antifolate to the tumor.

[0159] The amount of liposome pharmaceutical composition administered will depend upon the particular polyglutamated antifolate therapeutic entity entrapped inside the liposomes, the disease state being treated, the type of liposomes being used, and the judgment of the clinician. Generally the amount of liposome pharmaceutical composition administered will be sufficient to deliver a therapeutically effective dose of the particular therapeutic entity.

[0160] The quantity of liposome pharmaceutical composition necessary to deliver a therapeutically effective dose can be determined by routine *in vitro* and *in vivo* methods, common in the art of drug testing. See, for example, D. B. Budman, A. H. Calvert, E. K. Rowinsky (editors). Handbook of Anticancer Drug Development, LWW, 2003. Therapeutically effective dosages for various therapeutic compositions are known to those skilled in the art. In some embodiments, a therapeutic entity delivered via the pharmaceutical liposome composition provides at least the same or higher activity than the activity obtained by administering the same amount of the therapeutic entity in its routine non-liposome formulation. Typically the dosages for the liposome pharmaceutical composition is in a range for example, between about 0.005 and about 500 mg of the therapeutic entity per kilogram of body weight, most often, between about 0.1 and about 100 mg therapeutic entity/kg of body weight.

[0161] As used herein an "effective amount" refers to a dosage of an agent sufficient to provide a medically desirable result. The effective amount will vary with the desired outcome, the particular condition being treated or prevented, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent or combination therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose. In this disclosure, the term "subject" and "patient" is used interchangeably and has the same meaning. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment.

[0162] For example, if the subject has a tumor, an effective amount may be that amount of the agent (e.g., polyglutamated antifolate) that reduces the tumor volume or load (as for example determined by imaging the tumor). Effective amounts can also routinely be assessed by the presence and/or frequency of cancer cells in the blood or other body fluid or tissue (e.g., a biopsy). If the tumor is impacting the normal functioning of a tissue or organ, then the effective amount can routinely be assessed by measuring the normal functioning of the tissue or organ. In some instances the effective amount is the amount required to lessen or eliminate one or more, and preferably all, symptoms.

[0163] Terms such as "treating," or "treatment," or "to treat" refer to both (a) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and (b) prophylactic or preventative measures that prevent and/or slow the development of a targeted disease or condition. Thus, subjects in need of treatment include those already with the cancer or condition; those at risk of having the cancer or condition; and those in whom the infection or condition is to be prevented. In certain embodiments, a subject is successfully "treated" according to the methods provided herein if the subject shows, e.g., total, partial, or transient amelioration or elimination of a symptom associated with the disease or condition (e.g., cancer, rheumatoid arthritis).

[0164] Pharmaceutical compositions comprising the provided polyglutamated antifolate compositions (e.g., liposomes containing a pentaglutamated or hexaglutamated antifolate) are also provided. Pharmaceutical compositions are sterile compositions that comprise a sample liposome and preferably antifolate(s), preferably in a pharmaceutically-acceptable carrier.

[0165] The term "delivery vehicle" refers generally to any compositions that acts to assist, promote or facilitate entry of polyglutamated antifolates into a cell, for example, viral sequences, viral material, or lipid or liposome formulations.

[0166] The term "pharmaceutically-acceptable carrier" refers to, for example, one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other subject.

[0167] The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which liposome compositions are combined to facilitate administration. The components of the pharmaceutical compositions are comingled in a manner that precludes interaction that would substantially impair their desired pharmaceutical efficiency. Suitable buffering agents include acetic acid and a salt (1-2% W/V); citric acid and a salt (1-3% W/V); boric acid and a salt (0.5-2.5% W/V); and phosphoric acid and a salt (0.8-2% W/V). Suitable preservatives include benzalkonium chloride (0.003-0.03% W/V); chlorobutanol (0.3-0.9% W/V); and parabens (0.01-0.25% W/V).

[0168] Unless otherwise stated herein, a variety of administration routes are available. The particular mode selected will depend, upon the particular active agent selected, the particular condition being treated and the dosage required for therapeutic efficacy. The provided methods can be practiced using any known mode of administration that is medically acceptable and in accordance with good medical practice. In some embodiments, the administration route is an injection. In further embodiments, the injection is by a parenteral route elected from an intramuscular, subcutaneous, intravenous, intraarterial, intraperitoneal, intraarticular, intraepidural, intrathecal, intravenous, intramuscular, or intra sternal injection. In some embodiments, the administration route is an infusion. In additional embodiments, the administration route is oral, nasal, mucosal, sublingual, intratracheal, ophthalmic, rectal, vaginal, ocular, topical, transdermal, pulmonary, or inhalation.

[0169] In some embodiments, PLPAs and/or targeted-PLPAs are prepared as an infusion composition, an injection composition, a parenteral composition, or a topical composition. In further embodiments, the injection includes one or more of: intraperitoneal injection, direct intratumor injection, intra-arterial injection, and intravenous injection, subcutaneous injection, intramuscular injection, delivery via transcutaneous and intranasal route. In a further embodiment, the PLPA and/or targeted-PLPA is a liquid solution or a suspension. However, solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection are also provided herein. In some embodiments, the targeted pegylated liposomal polyglutamated antifolate is formulated as an enteric-coated tablet or gel capsule according to methods known in the art.

[0170] In some embodiments, the targeted pegylated liposomal polyglutamated antifolate formulations are administered to a tumor of the central nervous system using a slow, sustained intracranial infusion of the liposomes directly into the tumor (e.g., a convection-enhanced delivery (CED)). See, Saito et al., Cancer Research 64:2572-2579 (2004); Mamot et al., J. Neuro-Oncology 68:1-9 (2004). In other embodiments, the formulations are directly applied to tissue surfaces. Sustained release, pH dependent release, and other specific chemical or environmental condition-mediated release administration of the pegylated liposomal polyglutamated antifolates (e.g., depot injections and erodible implants) are also provided. Examples of such release-mediating compositions are further described herein or otherwise known in the art.

[0171] For administration by inhalation, the compositions can be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, ichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit can be determined by providing a valve to deliver a metered amount.

[0172] When it is desirable to deliver the compositions systemically, they can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers. Pharmaceutical parenteral formulations include aqueous solutions of the ingredients. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Alternatively, suspensions of liposomes can be prepared as oil-based suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides.

[0173] Alternatively, the non-targeted or targeted pegylated liposomal polyglutamated antifolates can be in powder form or lyophilized form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0174] The provided compositions (e.g., polyglutamated antifolates and liposomes containing the polyglutamated antifolates) can also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[0175] The provided compositions have applications *in vivo*, *ex vivo* and *in vitro*. In some embodiments, the compositions have *in vitro* applications. *In vitro* use may include uses such as cell culturing and tissue engineering where selective treatment of a subpopulation of cells is desired. For example, during the culture of stem cells from a normal patient or a patient suffering from cancer, the cells can be treated with a sample composition or sample liposome as discussed to address cancerous subpopulations of cells. The cancerous subpopulation may arise because the donor originally has cancer or because the cells spontaneously transform during *in vitro* procedures.

[0176] In some embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating a disorder of the immune system (e.g., an autoimmune disease such as rheumatoid arthritis), the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having an autoimmune disease. In some embodiments, the disorder of the immune system is an

autoimmune disease. In further embodiments, the disorder of the immune system is rheumatoid arthritis. In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated pralatrexate (PTX). In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated pralatrexate (PTX). In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In additional embodiments, the delivery vehicle comprises a targeting moiety on its surface that specifically binds an antigen on the surface of a target cell of interest. In further embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG), SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG), SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98.

[0177] In some embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating cancer, the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having cancer. In some embodiments, the cancer is a cancer selected from the group consisting of: lung (e.g., non-small lung cancer), pancreatic, breast cancer, ovarian, lung, prostate, head and neck, gastric, gastrointestinal, colon, esophageal, cervical, kidney, biliary duct, gallbladder, and a hematologic malignancy (e.g., a leukemia or lymphoma). In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the

liposome is pegylated. In additional embodiments, the delivery vehicle comprises a targeting moiety on its surface that specifically binds an antigen on the surface of a target cell of interest. In further embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG), SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of

[0178] In some embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating lung cancer (e.g., non-small lung cancer), the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having lung cancer. In particular embodiments, the, the cancer is non-small cell lung cancer. In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In some embodiments, the liposome is untargeted. In additional embodiments, the delivery vehicle comprises a targeting moiety on its surface that specifically binds an antigen on the surface of a lung cancer (e.g., non-small cell lung cancer) cell. In further embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of Mucin 1, Folate receptor- δ , Nectin 4, NaPi2b, CD56, EGFR, and SC-16. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of Mucin 1, Folate receptor- δ , Nectin 4, NaPi2b, CD56, EGFR, and SC-16.

[0179] In some embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating pancreatic cancer, the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having pancreatic cancer. In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hex-

aglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In some embodiments, the liposome is untargeted. In additional embodiments, the delivery vehicle comprises a targeting moiety on its surface that specifically binds an antigen on the surface of a pancreatic cancer cell. In further embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of TACSTD2 (TROP2), Mucin 1, Folate receptor- δ , Mesothelin, Guanylyl cyclase C (GCC), SLC44A4, and Nectin 4. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of TACSTD2 (TROP2), Mucin 1, Folate receptor- δ , Mesothelin, Guanylyl cyclase C (GCC), SLC44A4, and Nectin 4.

[0180] In additional embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating breast cancer (e.g., triple negative breast cancer (estrogen receptor-, progesterone receptor-, and HER2-)), the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having breast cancer. In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In some embodiments, the liposome is untargeted. In additional embodiments, the delivery vehicle comprises a targeting moiety on its surface that specifically binds an antigen on the surface of a breast cancer cell. In further embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of LIV-1 (ZIP6), EGFR, HER2, HER3, Mucin 1, Folate receptor- δ , GONMB, and Nectin 4. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of LIV-1 (ZIP6), EGFR, HER2, HER3, Mucin 1, Folate receptor- δ , GONMB, and Nectin 4.

[0181] In some embodiments, the provided compositions (e.g., liposomes containing polyglutamated antifolates) are administered to subjects having or at risk of having a hematological cancer that is distinguishable by the expression of a tumor specific antigen or tumor associated antigen on its cell surface. Thus, in some embodiments, the invention provides a targeting moiety and a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having a cancer, solid tumor, and/or metastasis that is distinguishable by the expression of a tumor specific antigen or tumor associated antigen on its cell surface cancer for use in a method for treating cancer, the use comprising administering an effective amount of the delivery vehicle (e.g., liposome) comprising a targeting moiety and a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having a cancer, solid tumor, and/or metastasis that is distinguishable by the expression of a tumor specific antigen or tumor associated antigen on its cell surface cancer, and wherein the targeting moiety is expressed on the surface of the delivery vehicle and specifically binds the tumor specific antigen or tumor associated antigen. In some embodiments, the administered

polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In additional embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen expressed on the surface of a hematological cancer cell. In additional embodiments, the targeting moiety specifically binds a cell surface antigen selected from the group consisting of CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98.

[0182] In some embodiments the disclosed compositions (e.g., liposomes containing polyglutamated antifolates) are administered to subjects having or at risk of having a cancer, a solid tumor, and/or a metastasis that is distinguishable by the expression of a tumor specific antigen or tumor associated antigen on its cell surface. Thus, in some embodiments, the invention provides a delivery vehicle (e.g., liposome) comprising a targeting moiety and a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate to a subject having or at risk of having a cancer, solid tumor, and/or metastasis that is distinguishable by the expression of a tumor specific antigen or tumor associated antigen on its cell surface cancer, and wherein the targeting moiety is expressed on the surface of the delivery vehicle and specifically binds the tumor specific antigen or tumor associated antigen for use in a method for treating cancer, the use comprising administering an effective amount of the delivery vehicle (e.g., liposome). In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises hexaglutamated PMX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. In additional embodiments, the delivery vehicle comprises a targeting moiety that specifically binds a cell surface antigen expressed on the surface of a cancer, a solid tumor, and/or a metastatic cell. In additional embodiments, targeting moiety specifically binds a cell surface antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG),

SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98. In further embodiments, the delivery vehicle is a liposome, and the liposome comprises a targeting moiety that specifically binds a cell surface antigen selected from the group consisting of GONMB, CD56, TACSTD2 (TROP2), CEACAM5, Folate receptor- α , Folate receptor- β , Mucin 1, Folate receptor- δ , STEAP1, Mesothelin, Nectin 4, ENPP3, Guanylyl cyclase C (GCC), SLC44A4, NaPi2b, CD70 (TNFSF7), CA9 (Carbonic anhydrase), 5T4 (TPBG), SLTRK6, SC-16, Tissue factor, LIV-1 (ZIP6), CGEN-15027, P-Cadherin, Fibronectin Extra-domain B (ED-B), VEGFR2 (CD309), Tenascin, Collagen IV, Periostin, endothelin receptor, HER 2, EGFR, CD30, CD79b, CD19, CD138, CD74, CD37, CD19, CD22, CD33, and CD98.

[0183] In further embodiments, the disclosure provides a delivery vehicle (e.g., liposome) comprising a targeting moiety on its surface the specifically binds a folate receptor, and a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate for use in a method for treating cancer, the use comprising administering an effective amount of the delivery vehicle to a subject having or at risk of having a cancer that contains cells expressing the folate receptor on their cell surface. In further embodiments, the folate receptor is folate receptor alpha, folate receptor beta or folate receptor delta. In some embodiments, the administered polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated AG2034, polyglutamated GW1843, polyglutamated aminopterin, and polyglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a pentaglutamated antifolate selected from the group consisting of pentaglutamated MTX, pentaglutamated PMX, pentaglutamated LTX, pentaglutamated AG2034, pentaglutamated RTX, pentaglutamated piritrexim, pentaglutamated pralatrexate, pentaglutamated AG2034, pentaglutamated GW1843, pentaglutamated aminopterin, and pentaglutamated LY309887. In some embodiments, the administered polyglutamated antifolate comprises a hexaglutamated antifolate selected from the group consisting of hexaglutamated MTX, hexaglutamated PMX, hexaglutamated LTX, hexaglutamated AG2034, hexaglutamated RTX, hexaglutamated piritrexim, hexaglutamated pralatrexate, hexaglutamated AG2034, hexaglutamated GW1843, hexaglutamated aminopterin, and hexaglutamated LY309887. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated PMX. In particular embodiments, the administered polyglutamated antifolate comprises pentaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated MTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated RTX. In some embodiments, the administered polyglutamated antifolate comprises pentaglutamated LTX. In some embodiments, the administered polyglutamated antifolate comprises hexaglutamated LTX. In some embodiments, the administered delivery vehicle is a liposome. In further embodiments, the liposome is pegylated. As disclosed herein, the folate receptor targeted pegylated liposomes containing polyglutamated antifolates are able to deliver high quantities of polyglutamated antifolates to cancer cells and particularly cancer cells that express folate receptors, compared to normal cells (*i.e.*, cells that unlike cancer cells do not actively take up liposomes, and/or do not express folate receptors). Any cancers that express folate receptors may be treated according to the disclosed methods. It should be noted that some cancers may express folate receptors in an early stage while the majority of cancers may express folate receptors at late stages.

[0184] Cancer that may be treated by the methods of the invention include carcinomas, sarcomas and melanomas. Carcinomas include without limitation to basal cell carcinoma, biliary tract cancer, bladder cancer, breast cancer, cervical cancer, choriocarcinoma, CNS cancer, colon and rectum cancer, kidney or renal cell cancer, larynx cancer, liver cancer, small cell lung cancer, non-small cell lung cancer (NSCLC, including adenocarcinoma, giant (or oat) cell carcinoma, and squamous cell carcinoma), oral cavity cancer, ovarian cancer, pancreatic cancer, prostate cancer, skin cancer (including basal cell cancer and squamous cell cancer), stomach cancer, testicular cancer, thyroid cancer, uterine cancer, rectal cancer, cancer of the respiratory system, and cancer of the urinary system.

[0185] Sarcomas are mesenchymal neoplasms that arise in bone (osteosarcomas) and soft tissues (fibrosarcomas). Sarcomas include without limitation liposarcomas (including myxoid liposarcomas and pleiomorphic liposarcomas), leiomyosarcomas, rhabdomyosarcomas, malignant peripheral nerve sheath tumors (also called malignant schwannomas, neurofibrosarcomas, or neurogenic sarcomas), Ewing's tumors (including Ewing's sarcoma of bone, extraskeletal (*i.e.*, not bone) Ewing's sarcoma, and primitive neuroectodermal tumor), synovial sarcoma, angiosarcomas, hemangiosarcomas, lymphangiosarcomas, Kaposi's sarcoma, hemangioendothelioma, desmoid tumor (also called aggressive fibromatosis), dermatofibrosarcoma protuberans (DFSP), malignant fibrous histiocytoma (MFH), hemangiopericytoma, malignant mesenchymoma, alveolar soft-part sarcoma, epithelioid sarcoma, clear cell sarcoma, desmoplastic small cell tumor, gastrointestinal stromal tumor (GIST), and chondrosarcoma.

[0186] Melanomas are tumors arising from the melanocytic system of the skin and other organs. Examples of melanoma include without limitation lentigomaligna melanoma, superficial spreading melanoma, nodular melanoma, and acral lentiginous melanoma.

[0187] In some embodiments, the cancer treated by one or more of the methods disclosed herein is a solid tumor lymphoma. Examples of solid tumor lymphoma include Hodgkin's lymphoma, Non-Hodgkin's lymphoma, and B cell lymphoma.

[0188] In some embodiments, the cancer treated by one or more of the methods disclosed herein is bone cancer, brain cancer, breast cancer, colorectal cancer, connective tissue cancer, cancer of the digestive system, endometrial cancer, esophageal cancer, eye cancer, cancer of the head and neck, gastric cancer, intra-epithelial neoplasm, melanoma neuroblastoma, Non-Hodgkin's lymphoma, non-small cell lung cancer, prostate cancer, retinoblastoma, or rhabdomyosarcoma.

[0189] The disclosed methods can practiced in any subject that is likely to benefit from delivery of compositions contemplated herein (e.g., polyglutamated antifolate compositions such as liposome containing a pentaglutamated or hexaglutamated antifolate). Mammalian subjects, and in particular, human subjects are preferred. In some embodiments, the subjects also include animals such as household pets (e.g., dogs, cats, rabbits, and ferrets), livestock or farm animals (e.g., cows, pigs, sheep, chickens and other poultry), horses such as thoroughbred horses, laboratory animals (e.g., mice, rats, and rabbits), and other mammals. In other embodiments, the subjects include fish and other aquatic species.

[0190] The subjects to whom the agents are delivered may be normal subjects. Alternatively the subject may have or be at risk of developing a condition that can be diagnosed or that can benefit from delivery of one or more of the provided compositions. In some embodiments, such conditions include cancer (e.g., solid tumor cancers or non-solid cancer such as leukemias). In some embodiments, these conditions (e.g., cancers) involve cells that express an antigen that can be specifically bound by a targeted pegylated liposomal polyglutamated antifolate disclosed herein. In further embodiments, these antigens specifically bind and internalize the targeted pegylated liposomal polyglutamated antifolate into the cell. In some embodiments, the targeted pegylated liposomal polyglutamated antifolate specifically binds a folate receptor (e.g., folate receptor alpha (FR- α), folate receptor beta (FR- β) and folate receptor delta (FR- δ)) expressed on the surface of the cancer cell.

[0191] Tests for diagnosing the conditions that can be treated with the provided compositions are known in the art and will be familiar to the medical practitioner. The determination of whether a cell type expresses folate receptors can be made using commercially available antibodies. These laboratory tests include without limitation microscopic analyses, cultivation dependent tests (such as cultures), and nucleic acid detection tests. These include wet mounts, stain-enhanced microscopy, immune microscopy (e.g., FISH), hybridization microscopy, particle agglutination, enzyme-linked immunosorbent assays, urine screening tests, DNA probe hybridization, and serologic tests. The medical practitioner will generally also take a full history and conduct a complete physical examination in addition to running the laboratory tests listed above.

[0192] A subject having a cancer can, for example, be a subject that has detectable cancer cells. A subject at risk of developing a cancer can, for example, be a subject that has a higher than normal probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality that has been demonstrated to be associated with a higher likelihood of developing a cancer, subjects having a familial disposition to cancer, subjects exposed to cancer causing agents (i.e., carcinogens) such as tobacco, asbestos, or other chemical toxins, and subjects previously treated for cancer and in apparent remission.

[0193] In some embodiments, the invention provides a method of making a liposomal composition disclosed herein. In one embodiment, the method includes forming a mixture comprising: (1) a liposomal component; and (2) a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate in aqueous solution. In further embodiments, the mixture comprises a pegylated liposomal component. The mixture is then homogenized to form liposomes in the aqueous solution. Further, the mixture can be extruded through a membrane to form liposomes enclosing the polyglutamated antifolate in an aqueous solution. It is understood the liposomal components of this disclosure can comprise any lipid (including cholesterol) including functionalized lipids and lipids attached to targeting moieties, detectable labels, and steric stabilizers, or any subset of all of these. It is further noted that the bioactive polyglutamated antifolate in aqueous solution can comprise any reagents and chemicals discussed herein or otherwise known in the art for the interior or exterior of the liposome including, for example, buffers, salts, and cryoprotectants.

[0194] In some embodiments, the disclosure provides a method of making a targeted pegylated liposomal polyglutamated antifolate (targeted-PLPA) or non-targeted PLPA disclosed herein. In one embodiment, the method includes forming a mixture comprising: (1) a liposomal component; (2) a polyglutamated (e.g., pentaglutamated or hexaglutamated) antifolate in aqueous solution; and (3) the targeting moiety. The mixture is then homogenized to form liposomes in the aqueous solution. Further, the mixture may be extruded through a membrane to form liposomes enclosing the targeted polyglutamated antifolate in an aqueous solution. It is understood that the targeted pegylated liposomal components can comprise any lipid (including cholesterol) including functionalized lipids and lipids attached to targeting moieties, detectable labels, and steric stabilizers, or any subset of all of these. It is further noted that the targeted pegylated liposome can comprise any reagents and chemicals discussed herein or otherwise known in the art for the interior or exterior of the liposome including, for example, buffers, salts, and cryoprotectants.

[0195] The above methods optionally further comprise the step of lyophilizing the composition after the removing step to form a lyophilized composition. As stated above, targeted-PTPLA or non-targeted-PTPLA in aqueous solution may

comprise a cryoprotectant described herein or otherwise known in the art. If the composition is to be lyophilized, a cryoprotectant may be preferred.

[0196] Additionally, after the lyophilizing step, the method optionally further comprises the step of reconstituting the lyophilized composition by dissolving the composition in a solvent after the lyophilizing step. Methods of reconstitution are known in the art. One example solvent is water. Other solvents include saline solutions and buffered solutions.

[0197] While certain example embodiments, are discussed herein, it is understood that liposomes can be made by any method that is known in the art. See, for example, G. Gregoriadis (editor), *Liposome Technology*, vol. 1-3, 1st edition, 1983; 2nd edition, 1993, CRC Press, 45 Boca Raton, Fla. Examples of methods suitable for making liposome compositions include extrusion, reverse phase evaporation, sonication, solvent (e.g., ethanol) injection, microfluidization, detergent dialysis, ether injection, and dehydration/rehydration. The size of liposomes can routinely be controlled by controlling the pore size of membranes used for low pressure extrusions or the pressure and number of passes utilized in microfluidization or any other suitable methods known in the art.

[0198] In general, the polyglutamated antifolate is contained inside, that is, in the inner (interior) space of the liposomes. In one embodiment, substituted ammonium is partially or substantially completely removed from the outer medium surrounding the liposomes. Such removal can be accomplished by any suitable means known in the art (e.g., dilution, ion exchange chromatography, size exclusion chromatography, dialysis, ultrafiltration, and precipitation). Accordingly, the methods of making liposomal compositions set forth above or otherwise known in the art can optionally further comprise the step of removing polyglutamated antifolate in aqueous solution outside of the liposomes after the extruding step.

[0199] In other embodiments, the disclosure provides a targeted pegylated liposomal polyglutamated antifolate (PLPA) that selectively targets folate receptors comprising: a liposome including an interior space, a polyglutamated antifolate disposed within the interior space, a steric stabilizer molecule attached to an exterior of the liposome, and a targeting moiety comprising a protein with specific affinity for at least one folate receptor, said targeting moiety attached to at least one of the steric stabilizer and the exterior of the liposome. The components of this embodiment may be the same as described for other embodiments, of this disclosure. For example, the targeted pegylated liposomal polyglutamated antifolate and the steric stabilizer which may be PEG, are as described in other parts of this disclosure.

[0200] In some embodiments the disclosure provides a method of preparing a targeted composition comprising a pegylated liposome including an entrapped and/or encapsulated polyglutamated antifolate; a targeting moiety an amino acid chain, the amino acid chain comprising a plurality of amino acids, the targeting moiety having a specific affinity for at least one type of folate receptor, the specific affinity being defined to include an equilibrium dissociation constant (K_d) in a range of 0.5×10^{-10} to 10×10^{-6} moles [0.05 nanoMole to 10 μ Mole] for at least one type folate receptor, the targeting moiety attached to one or both of a PEG and an exterior of the liposome, the method comprising: forming a mixture comprising: liposomal components; polyglutamated antifolate in solution; homogenizing the mixture to form liposomes in the solution; processing the mixture to form liposomes entrapping and/or encapsulating polyglutamated antifolate; and providing the targeting moiety on a surface of the liposomes entrapping and/or encapsulating polyglutamated antifolate, the targeting moiety having the specific affinity for at least one of folate receptor alpha ($FR-\alpha$), folate receptor beta ($FR-\beta$) and folate receptor delta ($FR-\delta$). In some embodiments the processing includes one or more of: thin film hydration, extrusion, in-line mixing, and stirring, and once the particles have been formed, the particles can have their sizes further modified by one or more of extrusion and sonication. In some embodiments, the targeted composition comprises at least 10% liposome entrapped polyglutamated antifolate. In some embodiments, the liposomes are anionic or neutral. In some embodiments, the targeting moiety has the specific affinity for one or more of: folate receptor alpha ($FR-\alpha$), folate receptor beta ($FR-\beta$) and folate receptor delta ($FR-\delta$). In further embodiments, the targeting moiety has the specific affinity for folate receptor alpha ($FR-\alpha$) and folate receptor beta ($FR-\beta$). In additional embodiments, the targeting moiety has the specific affinity for an epitope on a tumor cell surface antigen that is present on a tumor cell but absent or inaccessible on a non-tumor cell.

EXAMPLES

[0201] FIG. 5 shows chemical formula of L-gamma pentaglutamate pemetrexed. Once inside the cell, pemetrexed is converted to polyglutamate forms by the enzyme folypolyglutamate synthetase. The chemical formula shown is the pentaglutamated form where each glutamate is in the L-form. Further, each linkage is by the gamma carboxyl of the glutamate.

[0202] FIG. 6 shows chemical formulae of example L-gamma polyglutamated antifolate compositions encompassed by the disclosure. The glutamates are in the L-form and they are linked by the gamma carboxyl of glutamate.

METHODS

Production of Hexaglutamated Pemetrexed (HGP) Liposomes

[0203] Briefly Gamma HGP (gG6) was encapsulated in liposomes by the following procedure. First, the lipid components of the liposome membrane were weighed out and combined as a concentrated solution in ethanol at a temperature of around 65°C. In this example, the lipids used were hydrogenated soy phosphatidylcholine, cholesterol, and DSPE-PEG-2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]). The molar ratio of HSPC: Cholesterol: PEG-DSPE was approximately 3:2:0.15. Next, gG6 was dissolved in an aqueous buffer at a concentration of 20 mg/ml with a pH of 6.5-6.9. The drug solution was heated up to 65°C. The ethanolic lipid solution was injected into the gG6 solution using a small bore needle. During this step the drug solution was well stirred using a magnetic stirrer. The mixing was performed at an elevated temperature (63°C -72°C) to ensure that the lipids were in the liquid crystalline state (as opposed to the gel state that they attain at temperatures below the lipid transition temperature $T_m = 51^\circ\text{C}$ -54°C). As a result, the lipids were hydrated and form multiple bilayer (multilamellar) vesicles (MLV) containing gG6 in the aqueous core.

Downsizing of MLV's Using Filter Extrusion

[0204] The MLVs were fragmented into unilamellar (single bilayer) vesicles of the desired size by high-pressure extrusion using two passes through stacked (track-etched polycarbonate) membranes. The stacked membranes had two layers with a pore size of 200nm and six layers with a pore size of 100nm. During extrusion, the temperature was maintained above the T_m to ensure plasticity of the lipid membranes. As a result of the extrusion, large and heterogeneous in size and lamellarity MLVs turned into small, homogenous (100-120 nm) unilamellar vesicles (ULV) that sequestered the drug in their interior. A Malvern Zetasizer Nano ZS instrument (Southborough, MA) with back scattering detector (90°) was used for measuring the hydrodynamic size (diameter) at 25°C in a quartz micro cuvette. The samples were diluted 50-fold in formulation matrix before analysis.

Purification of liposomes

[0205] After the ULV's containing gG6 had been produced, the extra-liposomal gG6 was removed using columns for small volume or tangential flow diafiltration against a suitable buffer for large volume. Although any buffer solution can be used, in this example the buffer used was 5 mM HEPES, 145 mM Sodium Chloride, pH 6.7. Upon completion of purification, filter sterilization was performed using a 0.22 micron filter. Antibody conjugation

[0206] Activated liposomes were prepared by adding DSPE-PEG-maleimide to the lipid composition. The liposomes contain four different lipids: hydrogenated soy phosphatidylcholine (HSPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG-2000), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG-maleimide), in ratios of 3:2:0.1125:0.0375.

[0207] Antibody thiolation was accomplished through use of Traut's reagent (2-iminothiolane) to attach a sulfhydryl group onto primary amines. Antibody was suspended in PBS at a concentration of 0.9-1.6 mg/ml. Traut's reagent (14 mM) was added to antibody solution at a final concentration of 1-5 mM and then removed through dialysis after one-hour incubation at room temperature. Thiolated antibody was added to activated liposome at a ratio of 60 g/mol phosphate lipids, and the reaction mixture was incubated for one hour at room temperature and over-night at 4uL-cysteine was used to terminate the reaction and unconjugated antibody was removed through dialysis.

Physical Characteristics of the Nanoparticles**[0208]**

	Starting con.	Encapsulation efficiency	Final con.	Drug/ Lipid Ratio	Diameter	PDI	Zeta potential
Lps gG6	20 mg/ml	10.60%	1.39 mg/ml	35-50 g/mM lipids	114.9 nm	0.035	-1.76 mV

Dose response study of HGP (pentaglutamated pemetrexed) and liposomes.

[0209] Cell viability was determined by CellTiter-Glo®(CTG) luminescent cell viability assay on Day 3 (48 hour) and Day 4 (72 hour). This assay determines the number of viable cells in culture based on quantifying ATP that was present within, which in turn signals the presence of metabolically active cells. The CTG assay uses luciferase as a readout. To assess cell

viability Dose response inhibition of pemetrexed, HGP and liposomes on different cancer cell growth were investigated using CellTiterGlo® luminescent cell viability assay. Human cancer cells were harvested, counted and plated at a same cell density on Day 0. A series of 8 dilutions of each test article were added to the cells on Day 1. Dose response curve were generated and fit using GraphPad Prism and IC50 of each test article were calculated. A lower the IC50 is, the more potent the test article was in term of cancer cell growth inhibition.

[0210] Cells were seeded into 96-well plate at a cell density of 5×10^4 cells per well in 100 μ l of fresh media on Day 0. Eight serial 2-fold dilutions of each test article in culture medium were generated and added to cells in triplicate on Day 1. In addition, three wells of cells were treated with vehicle (HBS for free drug or empty liposome for liposomal HGP) alone as a control.

[0211] On Days 3 and 4, 100 μ l of CellTiterGlo® Reagent were added to each well and incubated at room temperature for 15 minutes. Luciferase luminescence were recorded for each well. In addition, 8 serial 2-fold dilutions of the vehicle (HBS or empty liposome) in culture medium were added into empty wells and included in the assay to generate the background luminescence signals. Luciferase signals were normalized by subtracting the background luminescence signal out of the read-outs respectively.

[0212] Human Normal Primary Bone Marrow CD34+ Cells were obtained from ATCC. (ATCC Catalog Number PCS-800-012). Cells were thawed at 37°C for 1 minute and then placed on ice. The cells were then resuspended in StemSpan SFEM (Stem Cell Tech Catalog Number 9650) plus 10% heat inactivated fetal bovine serum (Corning 35-015-CV). The cells were plated into 96 well culture plates at a density of 2.5×10^4 cells/well. The following day, live cells were collected via centrifugation and resuspended in neutrophil growth media (StemSpan SFEM plus 10% Heat Inactivated fetal bovine serum plus 100 ng/ml human stem cell factor (Sigma Catalog Number H8416), 20ng/ml human granulocyte colony-stimulation factor (Sigma Catalog Number H5541), and 10ng/ml human recombinant IL3 (Sigma SRP3090) at a density of 2.5×10^4 cells/well. Cells were incubated at 37°C for 10 days. Fresh media was added every two days. Mature neutrophils were then collected and plated in 96 well plates at a density of 1×10^4 cells/well and incubated at 37°C overnight. The next day, test article or vehicle was resuspended in neutrophil growth media and added to the plates. The cells were then incubated for either 48 hours or 72 hours at 37°C and then assayed at each time point using the Cell Titer Glo Assay (Promega Catalog #G7572).

[0213] Methodologies used for cell line AML 12 (non-cancerous liver cells) and CCD841 (non-cancerous colon epithelial cells) are similar to the methods used for cancer cells.

RESULTS

[0214] The dose response relationship of free pemetrexed gamma hexaglutamate (gG6), (untargeted) liposomal gamma hexaglutamate (liposomal gG6), pemetrexed and folate receptor alpha targeting antibody (FR1Ab) liposomal pemetrexed gamma hexaglutamate (liposomal gG6-FR1Ab), in the NCI H2342 non-small cell lung cancer (NSCLC), adenocarcinoma subtype is shown in **FIG 7**. The output is cell viability as measured by luciferase luminescence. As shown in this **FIG. 7**, the free pemetrexed gG6 appears to be the least potent as measured by IC50. Both the liposomal pemetrexed gG6 and the liposomal pemetrexed gG6-FR1Ab are 7-fold and 40-fold more potent, respectively, than free pemetrexed. **FIGS. 8 and 9** show similar data depicted as percentage of viable cells after 48 hours of treatment.

[0215] Similar data is shown for the HT-29 colon cancer cell line in **FIGS. 10 and 11** that depict cell viability expressed as a percentage. As shown in these figures, the free pemetrexed gG6 appears to be the least potent. In this instance, the liposomal pemetrexed gG6 is twice as potent as pemetrexed and the liposomal pemetrexed gG6-FR1Ab is 5-fold more potent than free pemetrexed.

[0216] Additional cell lines, namely OAW28 ovarian cancer cells and SW260 colon cancer cells, were also treated with pemetrexed γ G5 (also referred to as gG6) formulations. As shown in **FIG. 12**, and consistent with the data above, the free pemetrexed gG6 is the least effective and the liposomal pemetrexed gG6 appears to be more effective than pemetrexed.

[0217] **FIG. 13** shows that the liposomal pemetrexed gG6-FR1Ab leads to numerically higher inhibition of cancer cells than the non-targeted liposomal pemetrexed gG6 and the free pemetrexed gG6.

[0218] In another set of dose response experiments, 6 cell lines representing different types of cancers, namely HT-29 (colon cancer), H2342 (NSCLC, adenocarcinoma subtype), H292 (NSCLC, adenocarcinoma subtype), SW620 (CRC), H1806 (triple negative breast cancer) and OAW28 (ovarian cancer), were studied (**FIG. 14**). Treatment consisted of exposure for 48 hours using liposomal pemetrexed hexaaglutamate (liposomal gG6).

[0219] The relative potency of all of the above mentioned derivatives as compared to pemetrexed, following exposure over 48 hours, is represented in **FIG. 14**. The relative potency of treatment using the various derivatives, as shown in this figure was calculated by dividing the IC50 of pemetrexed by the IC50 of liposomal pemetrexed hexaaglutamate for each cell line. As shown in this figure, in all cell lines, the potency of liposomal pemetrexed hexaaglutamate well exceeded that of pemetrexed. By way of example, consider the NSCLC cell line H292. As shown in the figure, the potency of liposomal pemetrexed hexaglutamate ranged from 25-fold to ≥ 50 -fold that of pemetrexed. This suggests that a 4% or lower dose of the liposomal pemetrexed hexaglutamate would have the same treatment effect as a 100% dose of pemetrexed.

[0220] Cancer cell viability studies comparing liposomal pemetrexed hexaglutamate (liposomal gG6/Lps hexa gG6) and pemetrexed for cytotoxic activity on representative cell lines in breast, lung and ovarian cancer are shown in **FIGS. 15-17**. These data show that liposomal pemetrexed hexaglutamate is more potent than pemetrexed. Further, as an indicator of efficacy, the results of the experiments on the same cell lines depicted at various dose levels ranging from 16 to 128 nM in **FIGS. 18-20**. As shown in these figures, at each of these dose ranges, liposomal pemetrexed hexaglutamate is superior to pemetrexed in terms of inhibiting cancer cells for the lung and breast cancer cell lines (**FIGS. 18 and 19**, respectively). In the ovarian cancer cell line, pemetrexed at the dose of 128 nM, appears to be equally effective as liposomal pemetrexed hexaglutamate (see, **FIG. 20**, whereas the liposomal pemetrexed hexaglutamate at the dose of 32 nM and 64 nM has a better treatment effect than pemetrexed; at 16 nM the treatment effect is lower and similar in magnitude for liposomal pemetrexed hexaglutamate and pemetrexed.

[0221] The major toxicities seen in patients treated with pemetrexed is bone marrow suppression which manifests as a decrease in blood counts including neutrophil counts (a type of white blood cells). There is also some adverse effect on the lining of the mouth and gut that manifests as diarrhea and mucositis, as well as an adverse effect on the liver in some instances. To assess the above-mentioned toxicities, treatment liposomal pemetrexed hexaglutamate and pemetrexed was measured at 48 hours on CD34+ cells that were differentiated into neutrophils, CCD841 colon epithelium cells and AML12 liver cells. As shown in **FIG. 21**, liposomal pemetrexed hexaglutamate is significantly less toxic to differentiating human neutrophils in contrast to pemetrexed. This is also supported by neutrophil counts that are better preserved following treatment with the derivatives compared to pemetrexed, at dose ranges from 16 nM to 128 nM (**FIG. 21**). Strikingly, there does not appear to be any toxicity to the liver cells following treatment with liposomal pemetrexed hexaglutamate at the dose levels studied (**FIG. 22**). In contrast, pemetrexed at all doses studied is leading to a reduction in the liver cell counts of approximately 40%. And finally, the same trend is seen following treatment of epithelial colon cells (**FIG. 23**). As shown in this figure, pemetrexed at all doses studied is leading to approximately a $\geq 50\%$ decrease in the number of cells compared to approximately a 20% or less decrease after treatment with liposomal pemetrexed hexaglutamate.

Claims

1. A pegylated liposomal polyglutamated antifolate (LPA) composition comprising a pentaglutamated or hexaglutamated polyglutamated antifolate comprising L-glutamates linked by their gamma carboxyl groups, wherein the polyglutamated antifolate is a member selected from the group consisting of: polyglutamated methotrexate (MTX), polyglutamated pemetrexed (PMX), polyglutamated lometrexol (LTX), polyglutamated AG2034, polyglutamated raltitrexed (RTX), polyglutamated piritrexim, polyglutamated pralatrexate, polyglutamated GW1843, polyglutamated aminopterin, polyglutamated LY309887; optionally wherein the polyglutamated antifolate is polyglutamated PMX, MTX, RTX, or LTX.
2. The LPA composition according to claim 1, wherein the PEGylated liposome has a diameter in the range of 20 nm to 200 nm optionally in the range of 80 nm to 120 nm, and the liposome is formed from liposomal components comprising at least one of an anionic lipid, a neutral lipid, DSPE; DSPE-PEG-maleimide; HSPC; HSPC-PEG; cholesterol; cholesterol-PEG; and cholesterol-maleimide, and DSPE-PEG-FITC.
3. The LPA composition according to claim 2, wherein one or more liposomal components further comprises a steric stabilizer selected from the group consisting of polyethylene glycol (PEG); poly-L-lysine (PLL); monosialoganglioside (GM1); poly(vinyl pyrrolidone) (PVP); poly(acrylamide) (PAA); poly(2-methyl-2-oxazoline); poly(2-ethyl-2-oxazoline); phosphatidyl polyglycerol; poly[N-(2-hydroxypropyl) methacryl amide]; amphiphilic poly-N-vinylpyrrolidones; L-amino-acid-based polymer; and polyvinyl alcohol; optionally wherein said PEG has a number average molecular weight (Mn) of 200 to 5000 daltons.
4. The LPA composition according to any of the preceding claims, wherein the liposome is anionic, neutral, cationic, or has a zeta potential that is less than or equal to zero, optionally between 0 to -150 mV or, between -30 to -50 mV.
5. The LPA composition according to any of the preceding claims, wherein the liposome has an interior space and the interior space comprises the polyglutamated antifolate and an aqueous pharmaceutically acceptable carrier, optionally wherein the aqueous pharmaceutically acceptable carrier is trehalose, optionally wherein the pharmaceutically acceptable carrier comprises 5% to 20% weight of trehalose; optionally wherein the pharmaceutically acceptable carrier comprises citrate buffer at a concentration of between 5 to 200 mM and a pH of between 2.8 to 6; optionally wherein the pharmaceutically acceptable carrier comprises a total concentration of sodium acetate and calcium acetate of between 50 mM to 500 mM; optionally wherein the polyglutamated antifolate is at a pH of 5-8.

6. The LPA composition according to any of any of the preceding claims, wherein each PEGylated liposome comprises less than 200,000, optionally between 10,000 to 100,000, molecules of the polyglutamated antifolate.
7. The LPA composition according to any of any of the preceding claims, which further comprises a targeting moiety attached to one or both of a PEG and the exterior of the liposome, and wherein the targeting moiety has a specific affinity for a surface antigen on a target cell of interest, wherein the targeting moiety is attached to one or both of the PEG and the exterior of the liposome by a covalent bond, and wherein the targeting moiety is a polypeptide, an antibody, a humanized antibody, an antigen binding fragment of an antibody, a single chain antibody, a single-domain antibody, a bi-specific antibody, a synthetic antibody, a pegylated antibody, or a multimeric antibody.
8. The LPA composition according to claim 7, wherein the targeting moiety binds the antigen with an equilibrium dissociation constant (Kd) in a range of 0.5×10^{-10} to 10×10^{-6} as determined using BIACORE analysis; or wherein the targeting moiety specifically binds one or more folate receptors selected from the group consisting of: folate receptor alpha (FR- α), folate receptor beta (FR- β), and folate receptor delta (FR- δ), optionally wherein each PEGylated liposome comprises from 30 to 200 targeting moieties.
9. The LPA composition according to any of claims 7 or 8, further comprising one or more of an immunostimulatory agent, a detectable marker and a maleimide, wherein the immunostimulatory agent, the detectable marker or the maleimide is attached to said PEG or the exterior of the liposome; optionally wherein immunostimulating agent is at least one selected from the group consisting of: a protein immunostimulating agent; a nucleic acid immunostimulating agent; a chemical immunostimulating agent; a hapten; an adjuvant; a fluorescein; a fluorescein isothiocyanate (FITC); a DNP; a beta glucan; a beta-1,3-glucan; and a beta-1,6-glucan.
10. The LPA composition according to any of claims 1-9, which further comprises at least one cryoprotectant selected from the group consisting of mannitol; trehalose; sorbitol; and sucrose.
11. The LPA composition according to any of claims 1-10 for use in treating cancer, wherein the use comprises administering an effective amount of the liposomal polyglutamated antifolate composition of any of claims 1-10 to a subject having or at risk of having cancer; optionally wherein the cancer is selected from the group consisting of: lung cancer, pancreatic, breast cancer, ovarian cancer, lung cancer, prostate cancer, head and neck cancer, gastric cancer, gastrointestinal cancer, colon cancer, esophageal cancer, cervical cancer, kidney cancer, biliary duct cancer, gallbladder cancer, and a hematologic malignancy.
12. A method of preparing a liposomal polyglutamated antifolate (LPA) composition comprising the composition of any of claims 1-10, the method comprising: forming a mixture comprising liposomal components and polyglutamated antifolate in solution; homogenizing the mixture to form liposomes in the solution; and processing the mixture to form liposomes containing polyglutamated antifolate.

Patentansprüche

1. PEGylierte liposomale polyglutamierte Antifolat-(LPA)-Zusammensetzung, umfassend ein pentaglutamiertes oder hexaglutamiertes polyglutamiertes Antifolat, das durch ihre Gamma-Carboxylgruppen verknüpfte L-Glutamate umfasst, wobei das polyglutamierte Antifolat ein Mitglied ist, ausgewählt aus der Gruppe, bestehend aus: polyglutamiertem Methotrexat (MTX), polyglutamiertem Pemetrexed (PMX), polyglutamiertem Lometrexol (LTX), polyglutamiertem AG2034, polyglutamiertem Raltitrexed (RTX), polyglutamiertem Piritrexim, polyglutamiertem Pralatrexat, polyglutamiertem GW1843, polyglutamiertem Aminopterin, polyglutamiertem LY309887; wobei optional das polyglutamierte Antifolat polyglutamiertes PMX, MTX, RTX oder LTX ist.
2. LPA-Zusammensetzung nach Anspruch 1, wobei das PEGylierte Liposom einen Durchmesser im Bereich von 20 nm bis 200 nm, optional im Bereich von 80 nm bis 120 nm aufweist, und das Liposom aus liposomalen Komponenten gebildet ist, die mindestens eines von einem anionischen Lipid, einem neutralen Lipid, DSPE; DSPE-PEG-Maleimid; HSPC; HSPC-PEG; Cholesterin; Cholesterin-PEG; und Cholesterin-Maleimid, und DSPE-PEG-FITC umfassen.
3. LPA-Zusammensetzung nach Anspruch 2, wobei eine oder mehrere liposomale Komponenten ferner einen sterischen Stabilisator umfassen, der ausgewählt ist aus der Gruppe, bestehend aus Polyethylenglykol (PEG); Poly-L-Lysin (PLL); Monosialogangliosid (GM1); Poly(vinylpyrrolidon) (PVP); Poly(acrylamid) (PAA); Poly(2-methyl-2-oxazolin); Poly(2-ethyl-2-oxazolin); Phosphatidylpolyglycerin; Poly[N-(2-hydroxypropyl)methacrylamid]; amphiphi-

len Poly-N-vinylpyrrolidonen; L-aminosäurebasiertem Polymer; und Polyvinylalkohol; wobei optional das PEG ein zahlenmittleres Molekulargewicht (Mn) von 200 bis 5000 Dalton aufweist.

- 5 4. LPA-Zusammensetzung nach einem der vorhergehenden Ansprüche, wobei das Liposom anionisch, neutral, kationisch ist oder ein Zeta-Potenzial aufweist, das kleiner oder gleich null, optional zwischen 0 bis -150 mV oder zwischen -30 bis -50 mV ist.
- 10 5. LPA-Zusammensetzung nach einem der vorhergehenden Ansprüche, wobei das Liposom einen Innenraum aufweist und der Innenraum das polyglutamierte Antifolat und einen wässrigen pharmazeutisch annehmbaren Träger umfasst, wobei optional der wässrige pharmazeutisch annehmbare Träger Trehalose ist, wobei optional der pharmazeutisch annehmbare Träger 5 bis 20 Gew.-% Trehalose umfasst; wobei optional der pharmazeutisch annehmbare Träger Citratpuffer in einer Konzentration von zwischen 5 bis 200 mM und einem pH-Wert von zwischen 2,8 bis 6 umfasst; wobei optional der pharmazeutisch annehmbare Träger eine Gesamtkonzentration an Natriumacetat und Calciumacetat von zwischen 50 mM bis 500 mM umfasst; wobei optional das polyglutamierte Antifolat bei 15 einem pH-Wert von 5-8 vorliegt.
- 20 6. LPA-Zusammensetzung nach einem der vorhergehenden Ansprüche, wobei jedes PEGylierte Liposom weniger als 200.000, optional zwischen 10.000 bis 100.000 Moleküle des polyglutamierten Antifolats umfasst.
- 25 7. LPA-Zusammensetzung nach einem der vorhergehenden Ansprüche, die ferner eine an einem oder beiden von einem PEG und dem Äußeren des Liposoms befestigte Targeting-Einheit umfasst, und wobei die Targeting-Einheit eine spezifische Affinität für ein Oberflächenantigen auf einer Zielzelle von Interesse aufweist, wobei die Targeting-Einheit an einem oder beiden des PEG und dem Äußeren des Liposoms durch eine kovalente Bindung befestigt ist, und wobei die Targeting-Einheit ein Polypeptid, ein Antikörper, ein humanisierter Antikörper, ein Antigenbindungsfragment eines Antikörpers, ein Einzelkettenantikörper, ein Einzeldomänenantikörper, ein bispezifischer Antikörper, ein synthetischer Antikörper, ein pegylierter Antikörper oder ein multimerer Antikörper ist.
- 30 8. LPA-Zusammensetzung nach Anspruch 7, wobei die Targeting-Einheit das Antigen mit einer Gleichgewichtsdissoziationskonstante (K_d) in einem Bereich von $0,5 \times 10^{-10}$ bis 10×10^{-6} , wie bestimmt unter Verwendung von BIACORE-Analyse, bindet; oder wobei die Targeting-Einheit spezifisch einen oder mehrere Folatrezeptoren bindet, ausgewählt aus der Gruppe, bestehend aus: Folatrezeptor alpha (FR- α), Folatrezeptor beta (FR- β) und Folatrezeptor delta (FR- δ), wobei optional jedes PEGylierte Liposom 30 bis 200 Targeting-Einheiten umfasst.
- 35 9. LPA-Zusammensetzung nach einem der Ansprüche 7 oder 8, ferner umfassend eines oder mehrere von einem immunstimulierenden Mittel, einem detektierbaren Marker und einem Maleimid, wobei das immunstimulierende Mittel, der detektierbare Marker oder das Maleimid an dem PEG oder dem Äußeren des Liposoms befestigt ist; wobei optional das immunstimulierende Mittel mindestens eines ist, das aus der Gruppe ausgewählt ist, bestehend aus: einem immunstimulierenden Protein-Mittel; einem immunstimulierenden Nukleinsäure-Mittel; einem chemischen immunstimulierenden Mittel; einem Hapten; einem Adjuvans; einem Fluorescein; einem Fluoresceinisothiocyanat (FITC); einem DNP; einem Beta-Glucan; einem Beta-1,3-Glucan; und einem Beta-1,6-Glucan. 40
10. LPA-Zusammensetzung nach einem der Ansprüche 1-9, die ferner mindestens ein Gefrierschutzmittel umfasst, ausgewählt aus der Gruppe, bestehend aus Mannit; Trehalose; Sorbit; und Saccharose.
- 45 11. LPA-Zusammensetzung nach einem der Ansprüche 1-10 zur Verwendung beim Behandeln von Krebs, wobei die Verwendung Verabreichen einer wirksamen Menge der liposomalen polyglutamierten Antifolat-Zusammensetzung nach einem der Ansprüche 1-10 an ein Subjekt umfasst, das Krebs hat oder das Risiko hat, Krebs zu bekommen; wobei optional der Krebs ausgewählt ist aus der Gruppe, bestehend aus: Lungenkrebs, Bauchspeicheldrüsen-, Brustkrebs, Eierstockkrebs, Lungenkrebs, Prostatakrebs, Kopf-Hals-Krebs, Magenkrebs, Magen-Darm-Krebs, 50 Dickdarmkrebs, Speiseröhrenkrebs, Gebärmutterhalskrebs, Nierenkrebs, Gallengangkrebs, Gallenblasenkrebs und einer hämatologischen Malignität.
- 55 12. Verfahren zum Herstellen einer liposomalen polyglutamierten Antifolat-(LPA)-Zusammensetzung, umfassend die Zusammensetzung nach einem der Ansprüche 1-10, wobei das Verfahren Folgendes umfasst: Bilden einer Mischung, umfassend liposomale Komponenten und polyglutamiertes Antifolat in Lösung; Homogenisieren der Mischung, um Liposomen in der Lösung zu bilden; und Verarbeiten der Mischung, um Liposomen zu bilden, die polyglutamiertes Antifolat enthalten.

Revendications

1. Composition d'antifolate de polyglutamate liposomal pégylé (LPA) comprenant un antifolate de polyglutamate pentaglutamaté ou hexaglutamaté comprenant des L-glutamates liés par leurs groupes gamma carboxyle, dans laquelle l'antifolate de polyglutamate est un membre sélectionné parmi le groupe constitué de : méthotrexate de polyglutamate (MTX), pémétréxed de polyglutamate (PMX), lométrexol de polyglutamate (LTX), AG2034 de polyglutamate, raltitrexed de polyglutamate (RTX), piritrexim de polyglutamate, pralatrexate de polyglutamate, GW1843 de polyglutamate, aminoptérine de polyglutamate, LY309887 de polyglutamate ; éventuellement, dans laquelle l'antifolate de polyglutamate est du PMX, du MTX, du RTX ou du LTX de polyglutamate.
2. Composition de LPA selon la revendication 1, dans laquelle le liposome PEGylé a un diamètre compris entre 20 nm et 200 nm, éventuellement entre 80 nm et 120 nm, et le liposome est formé de composants liposomaux comprenant au moins l'un des éléments suivants : lipide anionique, lipide neutre, DSPE ; DSPE-PEG-maléimide ; HSPC ; HSPC-PEG ; cholestérol ; cholestérol-PEG ; et cholestérol-maléimide, et DSPE-PEG-FITC.
3. Composition de LPA selon la revendication 2, dans laquelle un ou plusieurs composants liposomaux comprennent en outre un stabilisant stérique sélectionné parmi le groupe constitué de : polyéthylène glycol (PEG) ; poly-L-lysine (PLL) ; monosialoganglioside (GM1) ; poly(vinylpyrrolidone) (PVP) ; poly(acrylamide) (PAA) ; poly(2-méthyl-2-oxazoline) ; poly(2-éthyl-2-oxazoline) ; phosphatidylpolyglycérol ; poly[N-(2-hydroxypropyl)méthacrylamide] ; poly-N-vinylpyrrolidones amphiphiles ; polymère à base d'acide L-aminé ; et alcool polyvinylique ; éventuellement dans laquelle ledit PEG a une masse moléculaire moyenne en nombre (Mn) de 200 à 5000 daltons.
4. Composition de LPA selon l'une quelconque des revendications précédentes, dans laquelle le liposome est anionique, neutre, cationique ou a un potentiel zêta inférieur ou égal à zéro, éventuellement entre 0 et -150 mV ou entre -30 et -50 mV.
5. Composition de LPA selon l'une quelconque des revendications précédentes, dans laquelle le liposome possède un espace intérieur et l'espace intérieur contient l'antifolate de polyglutamate et un excipient aqueux pharmaceutiquement acceptable, éventuellement dans laquelle l'excipient aqueux pharmaceutiquement acceptable est constitué de tréhalose, éventuellement dans laquelle l'excipient aqueux pharmaceutiquement acceptable comprend une concentration de 5 % à 20 % en poids de tréhalose ; éventuellement dans laquelle l'excipient aqueux pharmaceutiquement acceptable comprend un tampon citrate à une concentration comprise entre 5 et 200 mM et à un pH compris entre 2,8 et 6 ; éventuellement dans laquelle l'excipient aqueux pharmaceutiquement acceptable comprend de l'acétate de sodium et de l'acétate de calcium à une concentration totale comprise entre 50 mM et 500 mM ; éventuellement dans laquelle l'antifolate de polyglutamate est à un pH compris entre 5 et 8.
6. Composition de LPA selon l'une quelconque des revendications précédentes, dans laquelle chaque liposome PEGylé comprend moins de 200 000, éventuellement entre 10 000 et 100 000, molécules d'antifolate de polyglutamate.
7. Composition de LPA selon l'une quelconque des revendications précédentes, qui comprend en outre un fragment de ciblage attaché à un ou aux deux PEG et à l'extérieur du liposome, et dans laquelle le fragment de ciblage a une affinité spécifique pour un antigène de surface sur une cellule cible d'intérêt, dans laquelle le fragment de ciblage est attaché à un ou aux deux PEG et à l'extérieur du liposome par une liaison covalente, et dans laquelle le fragment de ciblage est un polypeptide, un anticorps, un anticorps humanisé, un fragment de liaison à l'antigène d'un anticorps, un anticorps à chaîne unique, un anticorps à domaine unique, un anticorps bispécifique, un anticorps synthétique, un anticorps pégylé ou un anticorps multimérique.
8. Composition de LPA selon la revendication 7, dans laquelle le fragment de ciblage se lie à l'antigène avec une constante de dissociation à l'équilibre (Kd) comprise entre $0,5 \times 10^{-10}$ et 10×10^{-6} déterminée par analyse BIACORE ; ou dans laquelle le fragment de ciblage se lie spécifiquement à un ou plusieurs récepteurs de folate sélectionnés parmi le groupe constitué de : récepteur de folate alpha (FR- α), récepteur de folate bêta (FR- β) et récepteur de folate delta (FR- δ), éventuellement dans laquelle chaque liposome PEGylé comprend de 30 à 200 fragments de ciblage.
9. Composition de LPA selon l'une quelconque des revendications 7 ou 8, comprenant en outre l'un ou plusieurs des éléments suivants : un agent immunostimulant, un marqueur détectable et un maléimide, dans laquelle l'agent immunostimulant, le marqueur détectable ou le maléimide sont fixés audit PEG ou à l'extérieur du liposome ;

éventuellement dans laquelle l'agent immunostimulant est au moins un agent sélectionné parmi le groupe constitué de : un agent immunostimulant protéique ; un agent immunostimulant d'acide nucléique ; un agent immunostimulant chimique ; un haptène ; un adjuvant ; une fluorescéine ; un isothiocyanate de fluorescéine (FITC) ; un DNP ; un bêta-glucane ; un bêta-1,3-glucane ; et un bêta-1,6-glucane.

5

10. Composition de LPA selon l'une quelconque des revendications 1 à 9, qui comprend en outre au moins un cryoprotecteur sélectionné parmi le groupe constitué de : mannitol ; tréhalose ; sorbitol ; et saccharose.

10

11. Composition de LPA selon l'une quelconque des revendications 1 à 10 destinée à être utilisée pour le traitement du cancer, dans laquelle l'utilisation comprend l'administration d'une quantité efficace de la composition d'antifolate de polyglutamate liposomal selon l'une quelconque des revendications 1 à 10 à un sujet atteint ou à risque d'être atteint d'un cancer ; éventuellement dans laquelle le cancer est sélectionné parmi le groupe constitué de : cancer du poumon, du pancréas, du sein, de l'ovaire, de la prostate, de la tête et du cou, de l'estomac, du système gastro-intestinal, du côlon, de l'œsophage, du col de l'utérus, du rein, des voies biliaires, de la vésicule biliaire et une hémopathie maligne.

15

12. Procédé de préparation d'une composition d'antifolate de polyglutamate liposomal (LPA) comprenant la composition de l'une quelconque des revendications 1 à 10, le procédé comprenant : la formation d'un mélange comprenant des composants liposomaux et de l'antifolate de polyglutamate en solution ; l'homogénéisation du mélange pour former des liposomes dans la solution ; et le traitement du mélange pour former des liposomes contenant de l'antifolate de polyglutamate.

20

25

30

35

40

45

50

55

FIG. 1

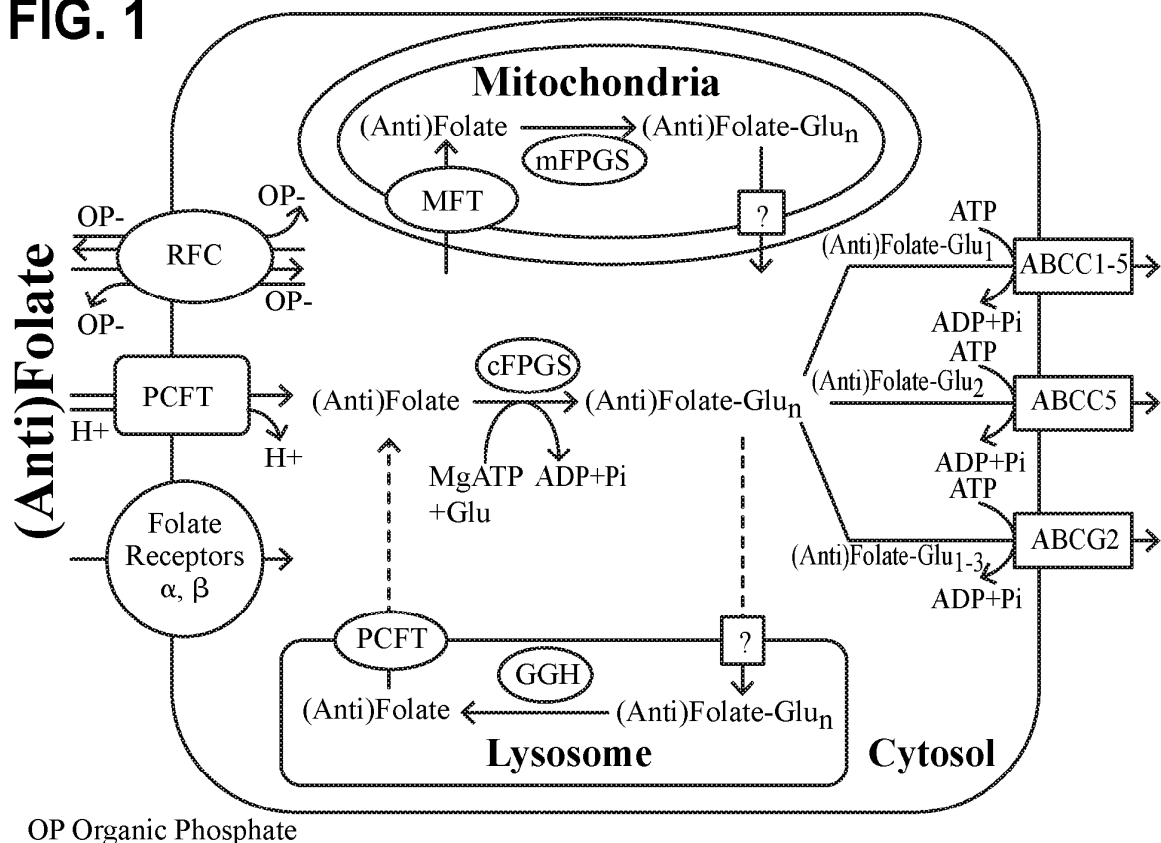
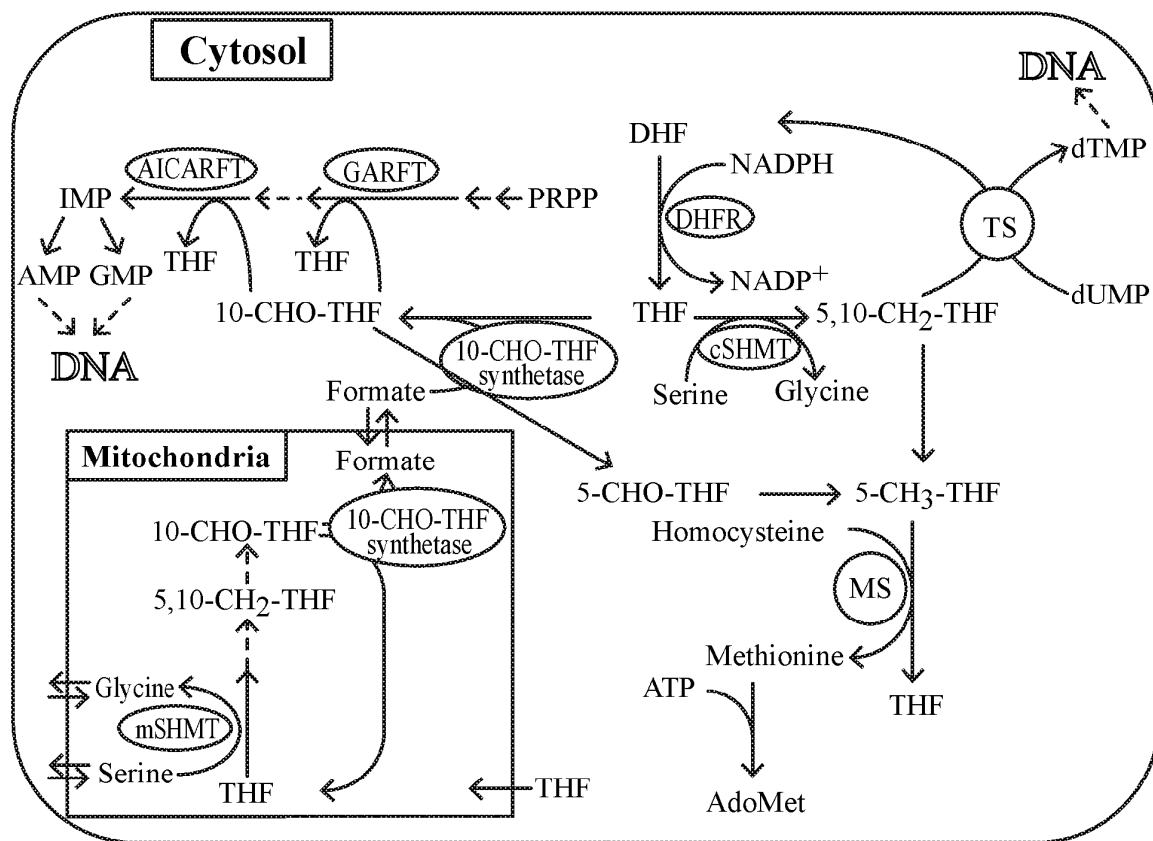


FIG. 2



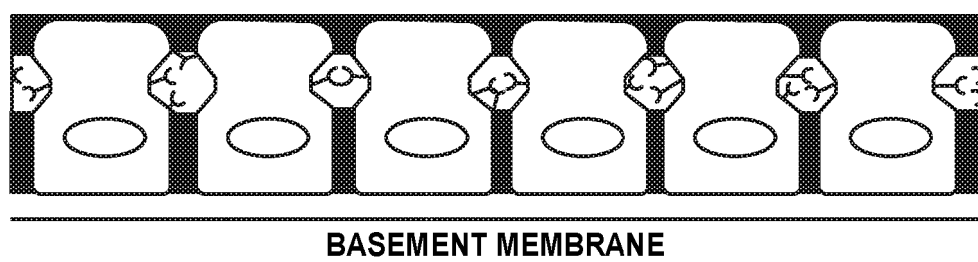


FIG. 3A

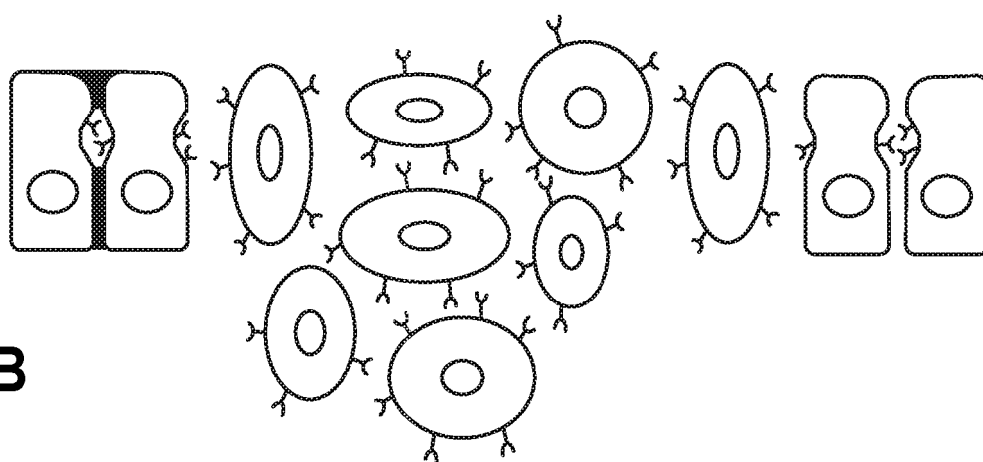
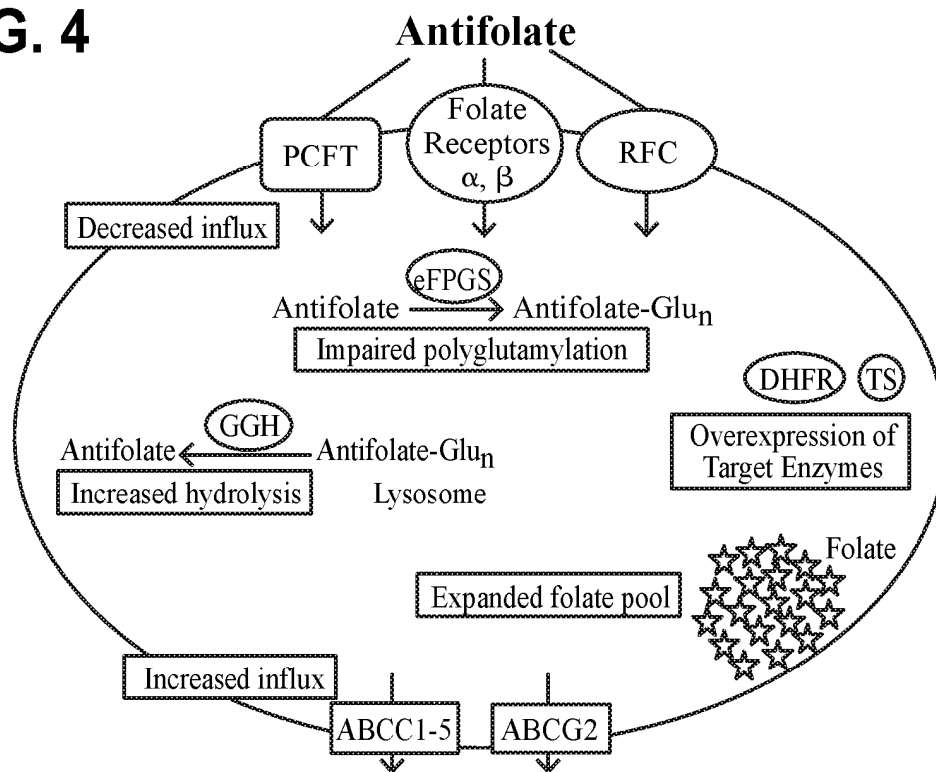
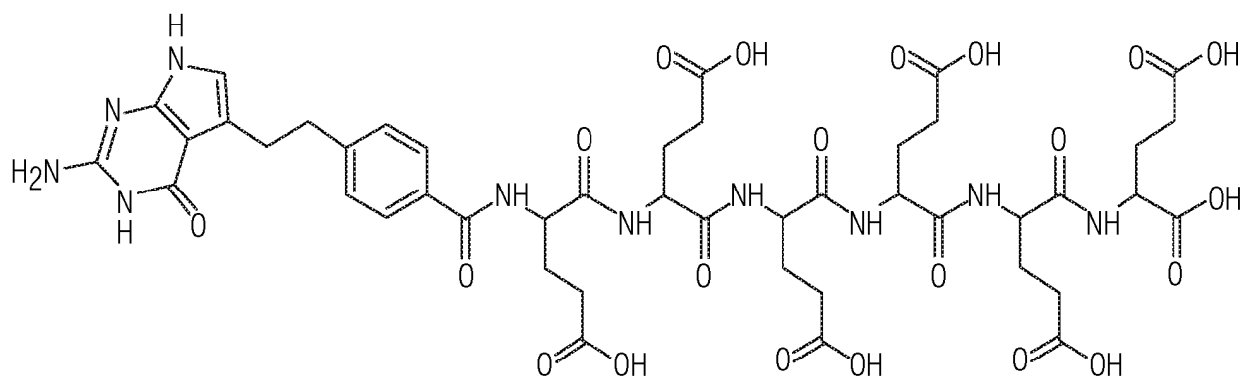


FIG. 3B

FIG. 4**FIG. 5**

Example L-gamma- polyglutamated antifolates

PMX-[L-glutamyl]_n

MTX-[L-glutamyl]_n

LTX-[L-glutamyl]_n

RTX-[L-glutamyl]_n

piritrexim-[L-glutamyl]_n

pralatrexate-[L-glutamyl]_n

AG2034-[L-glutamyl]_n

GW1843-[L-glutamyl]_n

aminopterin-[L-glutamyl]_n

LY309887-[L-glutamyl]_n

Wherein: the L-glutamyl groups are in gamma linkage (linked by their gamma carboxyl groups) and $n = 4, 5, 2-10, 4-6, \text{ or } >5$

FIG. 6

Dose Response Curve of Hexaglutamated Pemetrexed on NCI-H2342 at 48 Hours

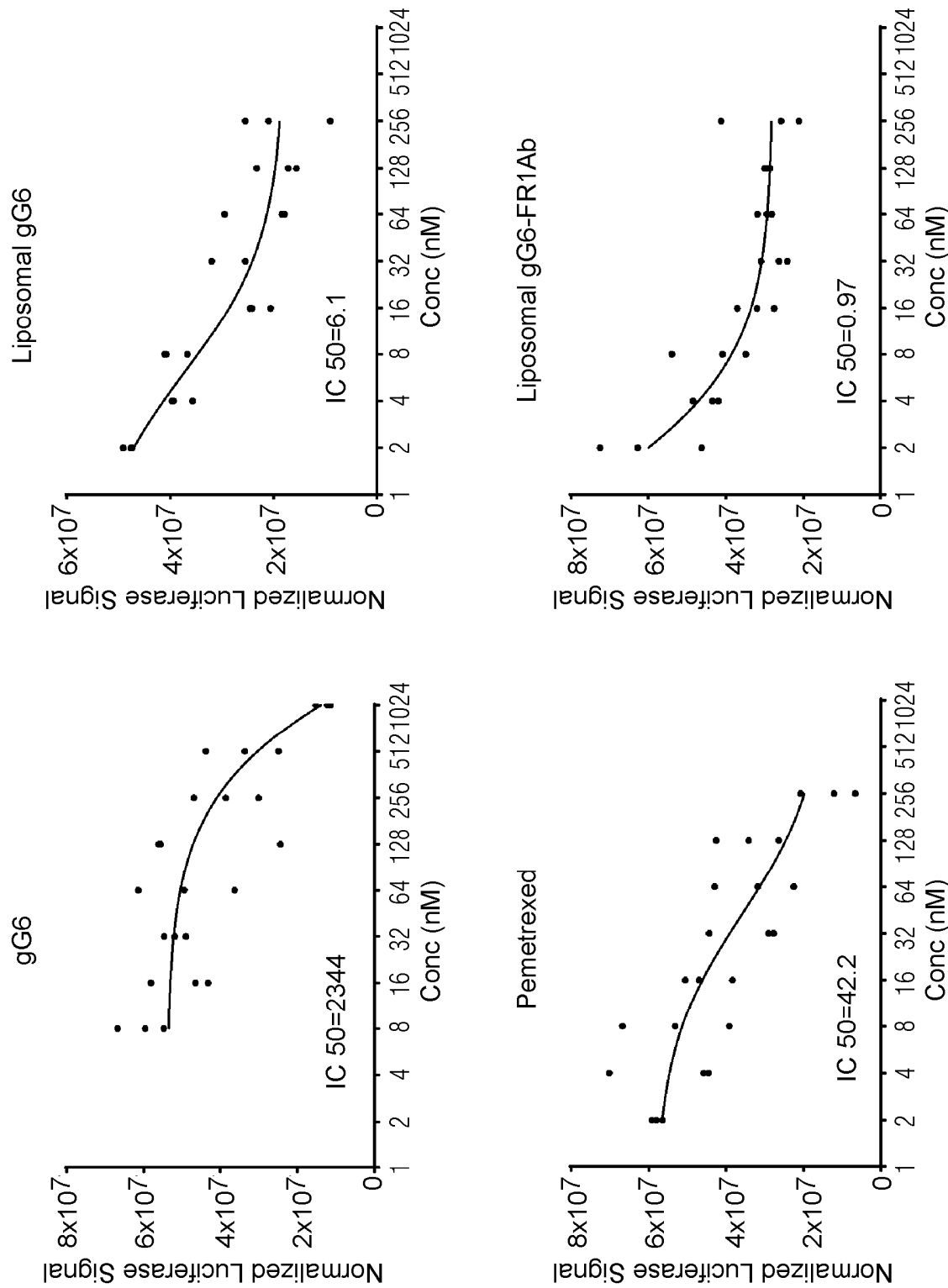


FIG. 7

Dose Response Curve of Hexaglutamated Pemetrexed on NCI-H2342 at 48 Hours

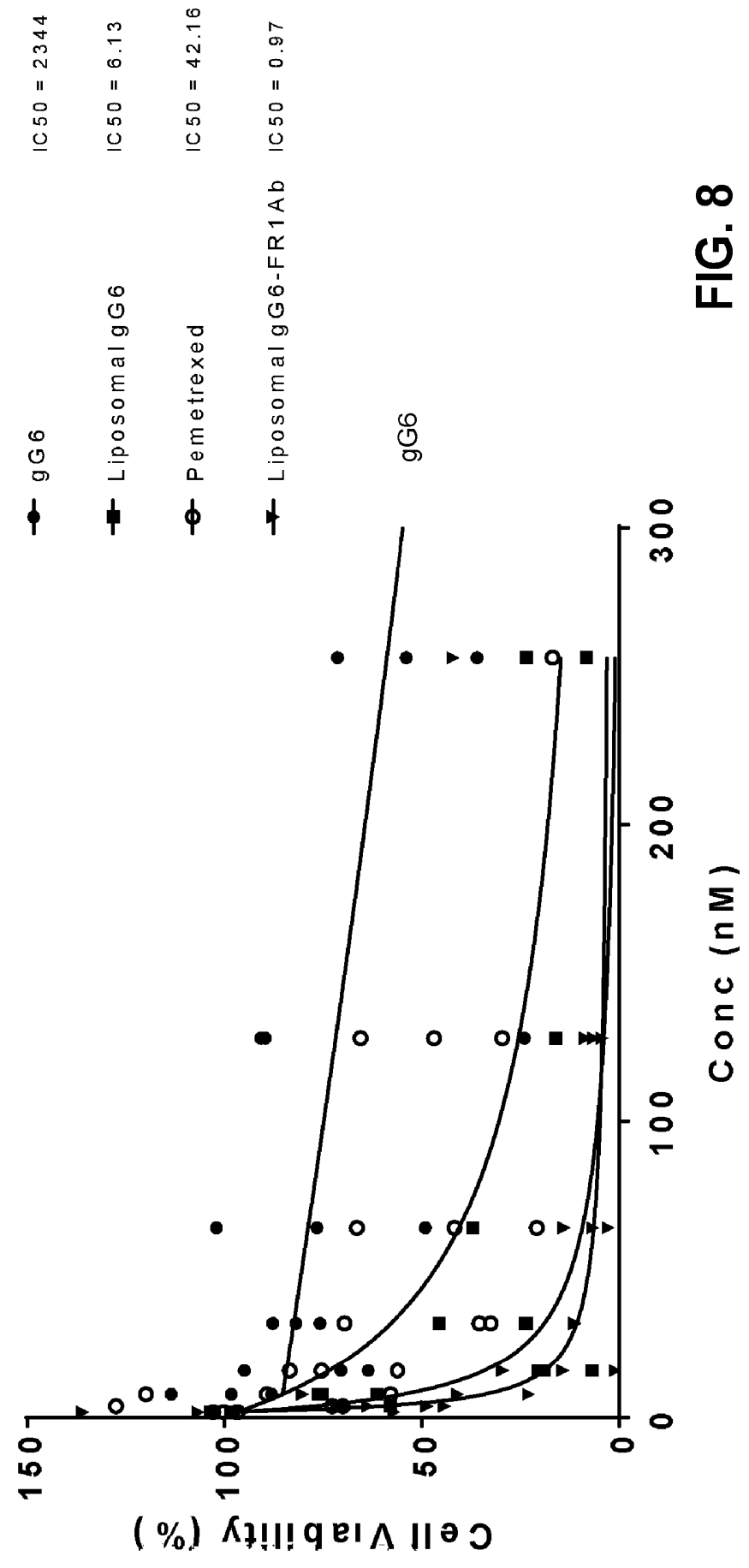


FIG. 8

Dose Response Curve of Hexaglutamated Pemetrexed on NCI-H2342 at 48 Hours

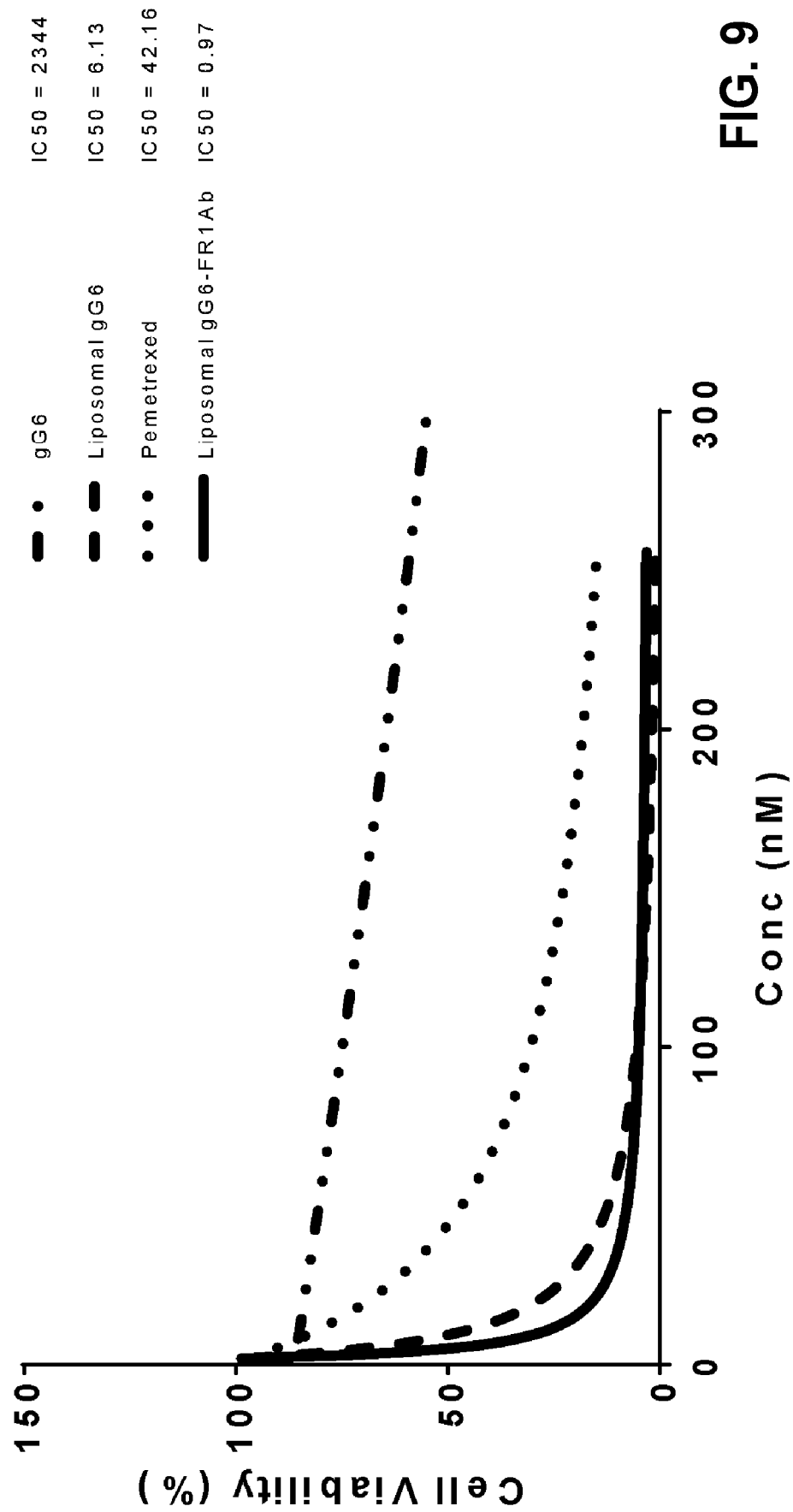


FIG. 9

Dose Response Curve of Hexaglutamated Pemetrexed on HT-29 (Colon Cancer)
at 48 Hours

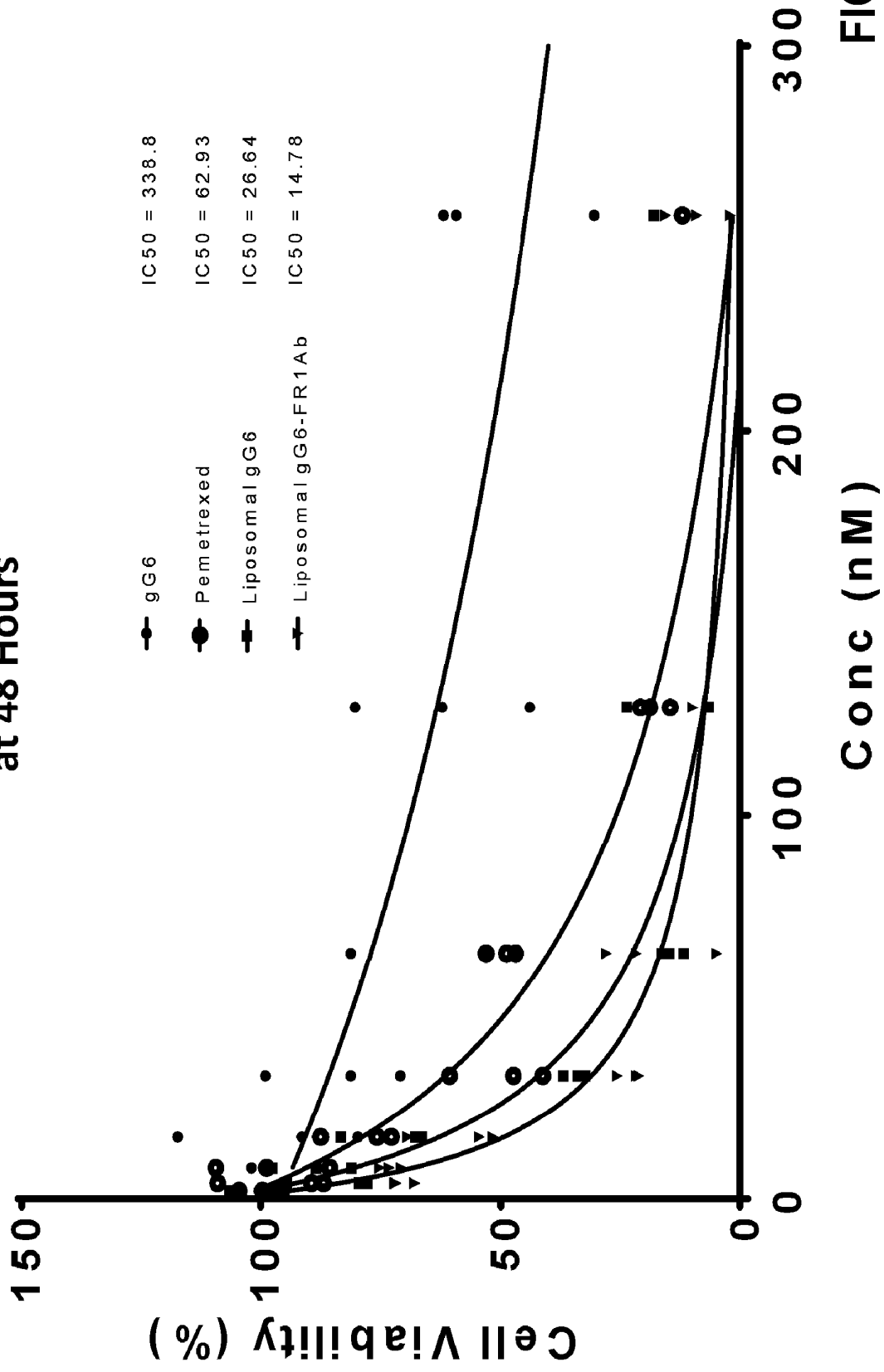


FIG. 10

Dose Response Curve of Hexaglutamated Pemetrexed on HT-29 (Colon Cancer) at 48 Hours

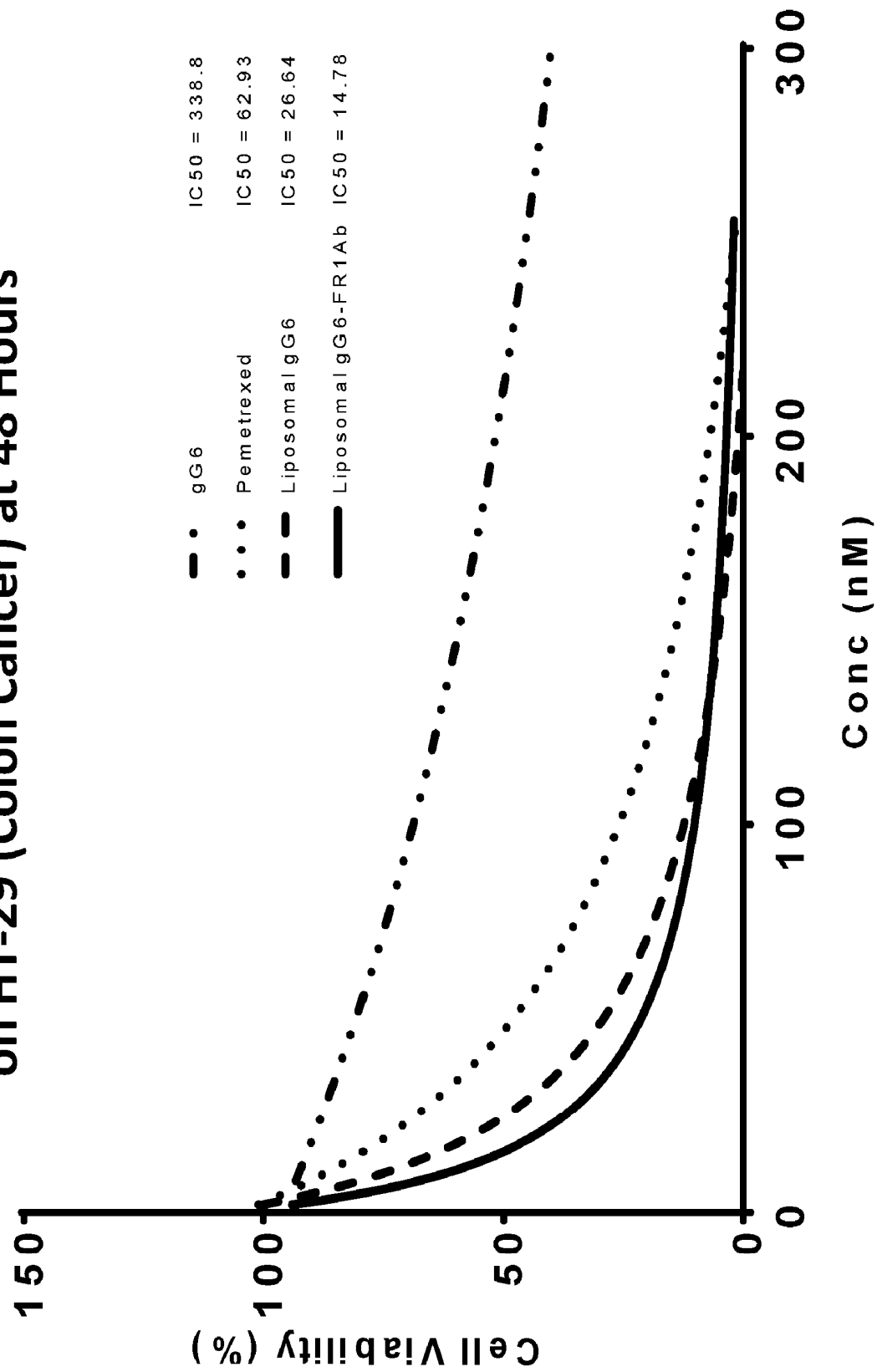


FIG. 11

Liposomal Hexa gG6 is able to enter cells more efficiently than Hexa gG6 and pemetrexed to inhibit growth (OAW28 cells)

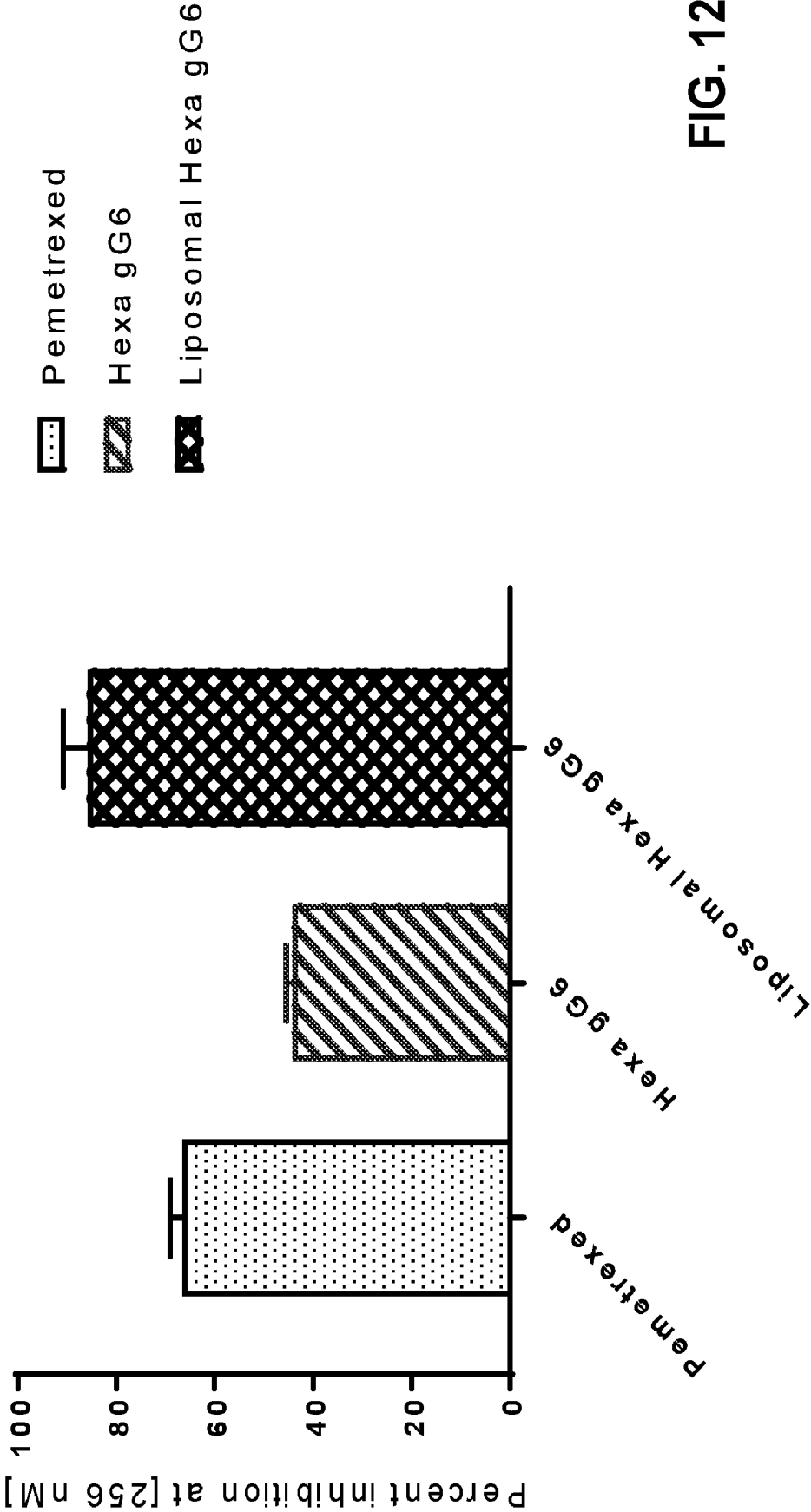


FIG. 12

Untargeted and targeted liposomal Hexa gG6 allows entry of Hexa gG6 into colon cancer cells (SW260)

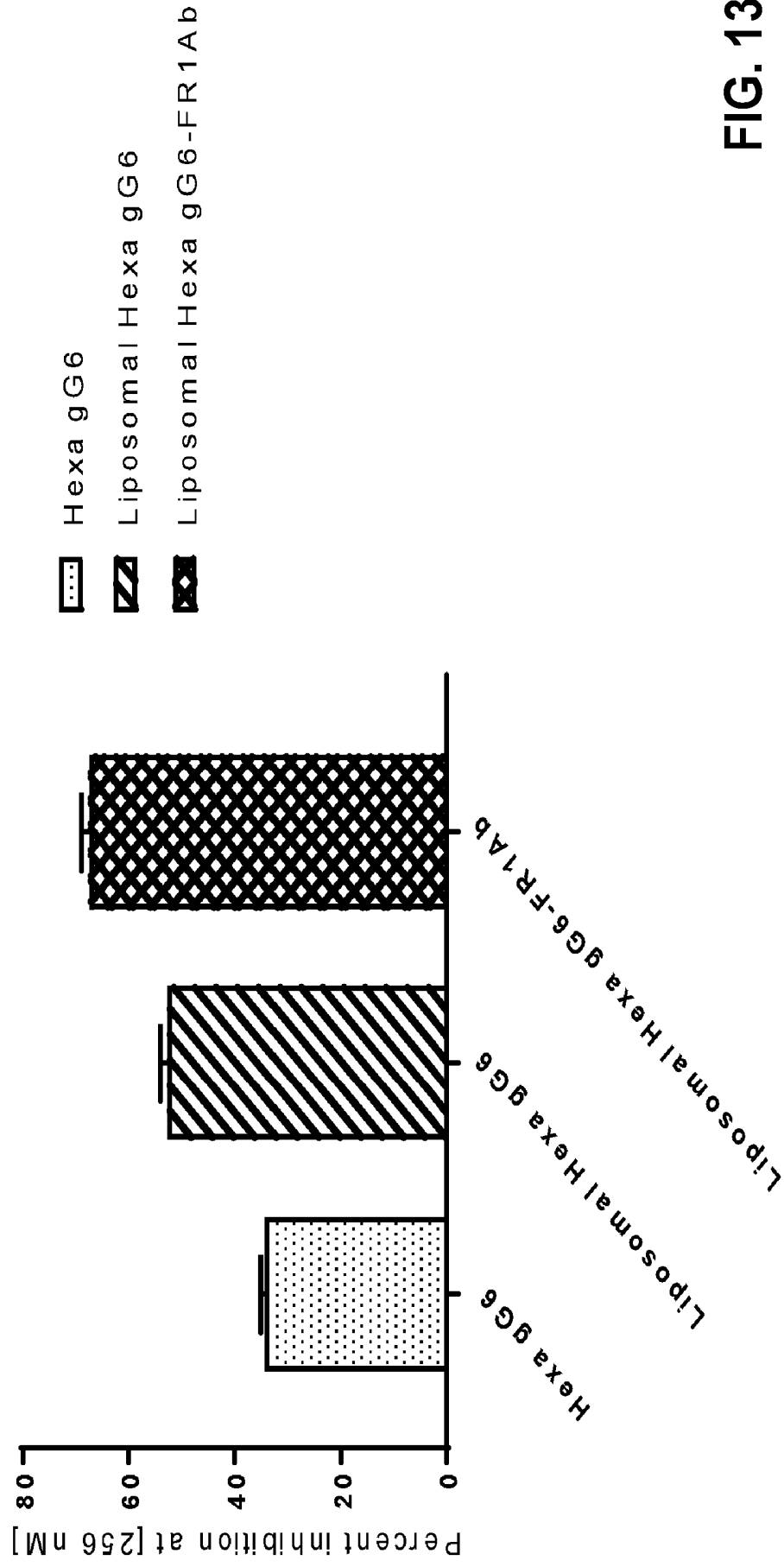


FIG. 13

Relative Potency of Liposomal L-Gamma Hexaglutamate Pemetrexed as Compared to Pemetrexed Following Cell Exposure Over 48 Hours

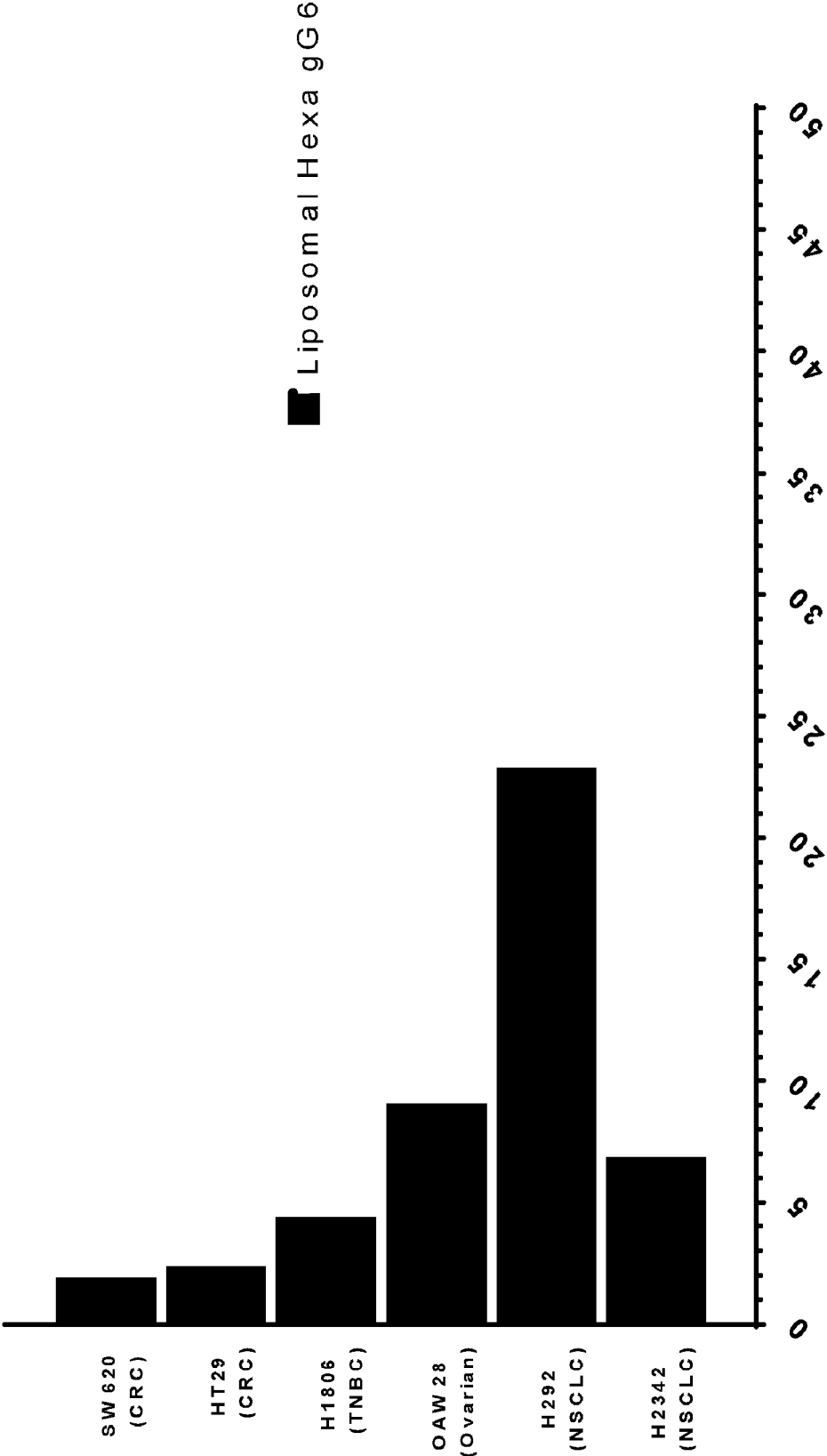


FIG. 14

Treatment Effect on HCC1806 Triple Negative Breast Cancer Cells Following Exposure Over 48 Hours

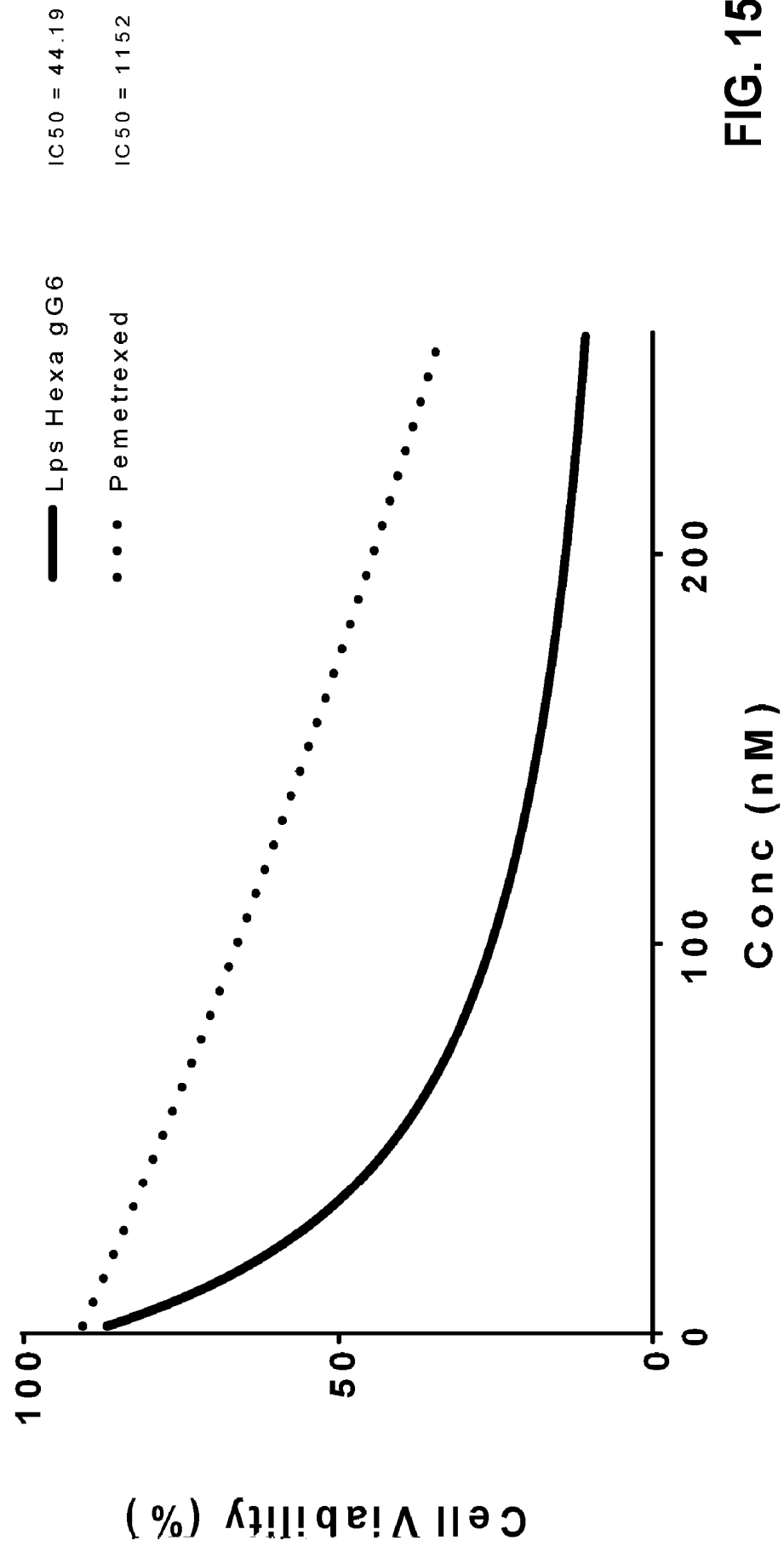


FIG. 15

Treatment Effect on H292 Non Small Cell Lung Cancer Cells Following Exposure Over 48 Hours

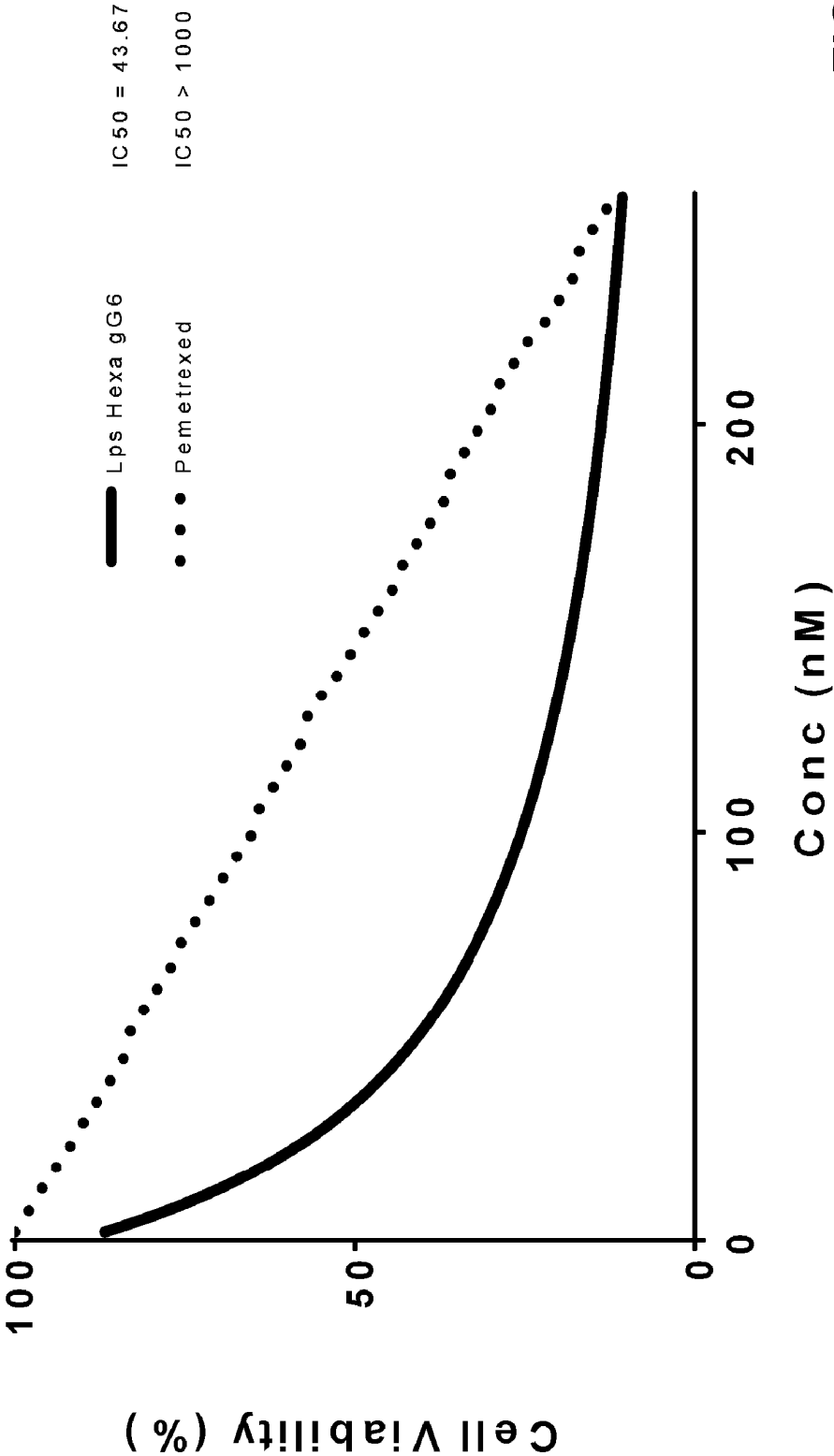


FIG. 16

Treatment Effect on OAW28 Ovarian Cancer Cells Following Exposure Over 48 Hours

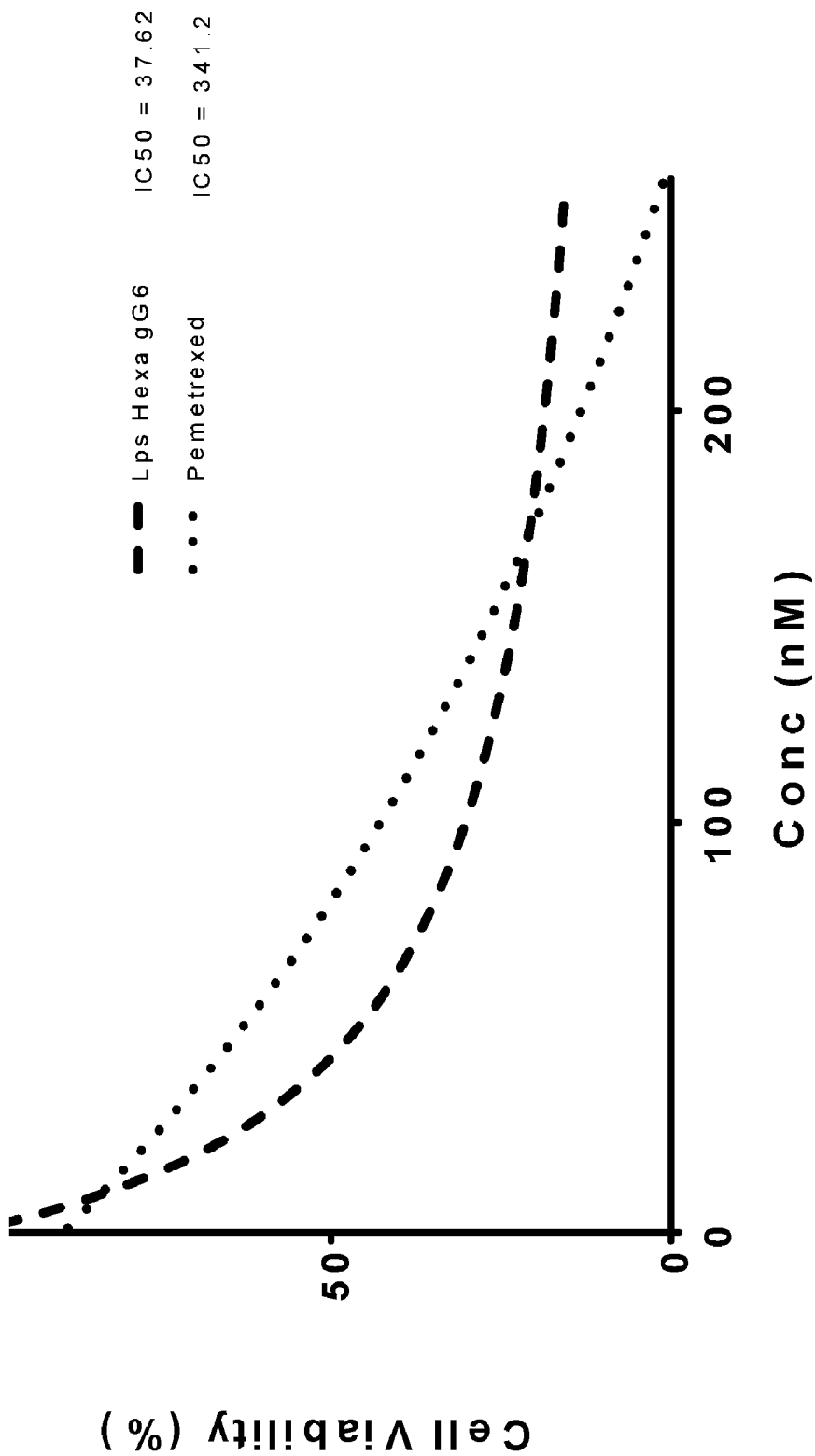


FIG. 17

Treatment Effect on H292 Non Small Cell Lung Cancer Cells
Following Exposure Over 48 Hours

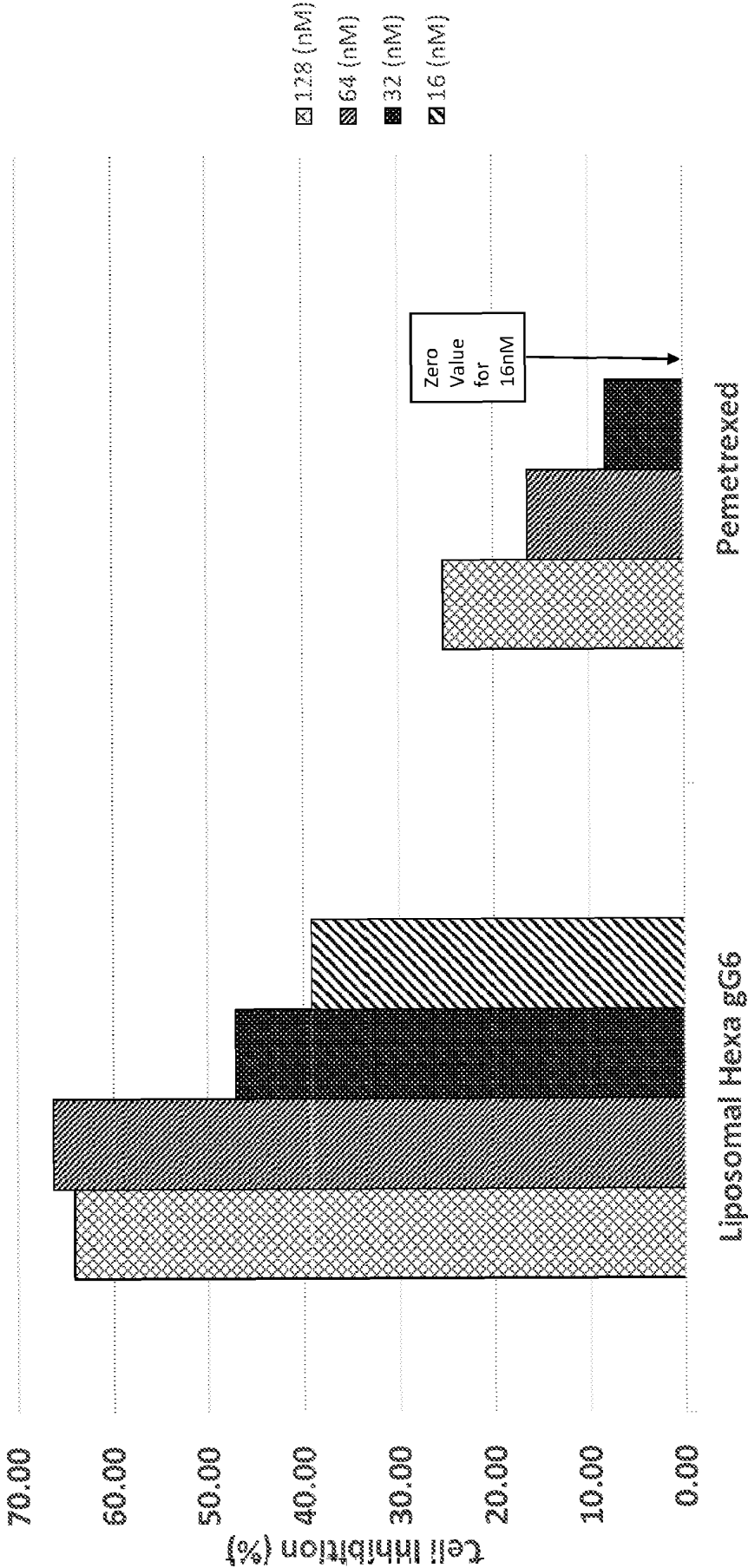


FIG. 18

HCC1806 Triple Negative Breast Cancer Cells Following Exposure Over 48 Hours

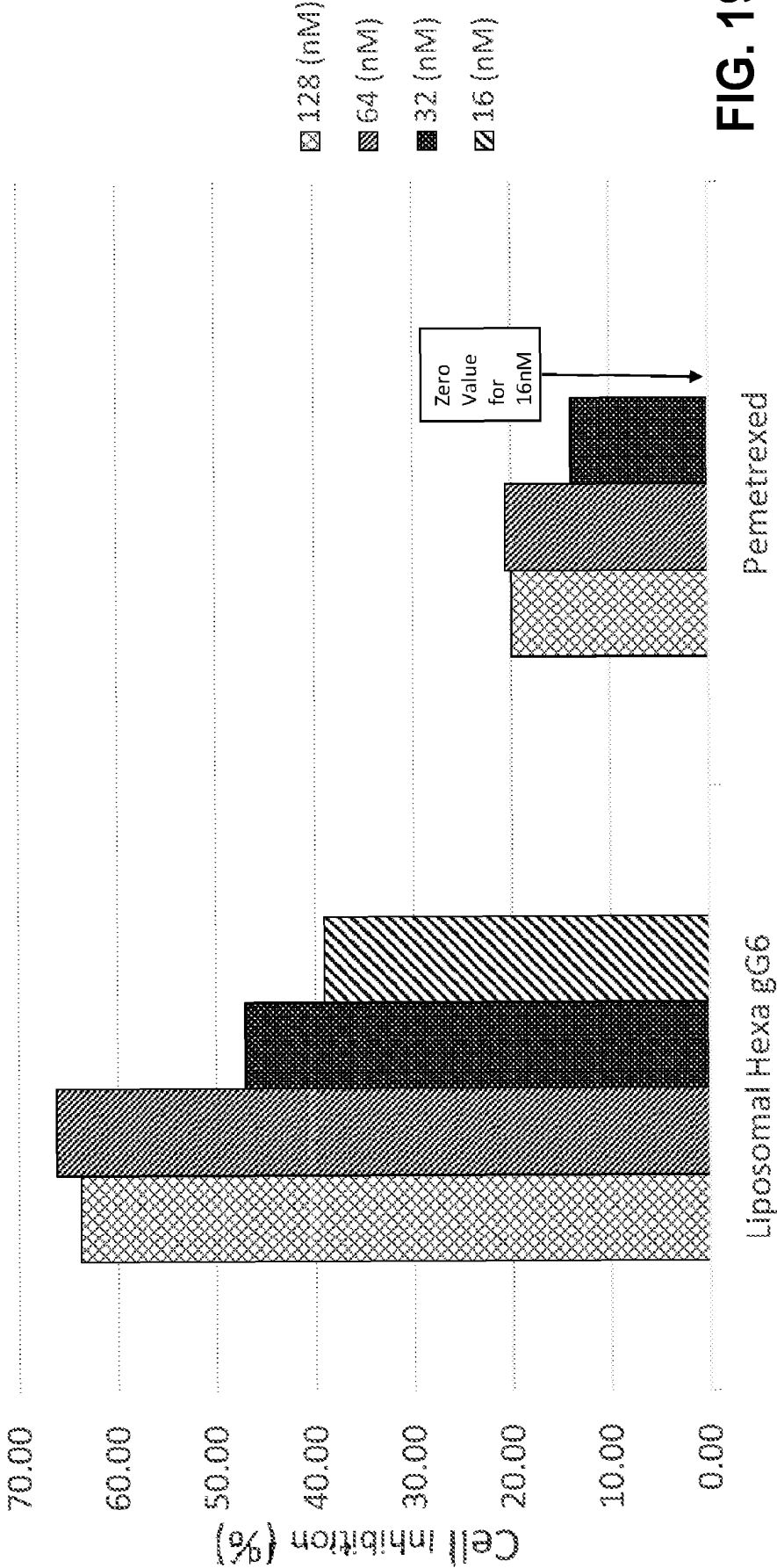


FIG. 19

Treatment Effect On OAW28 Ovarian Cancer Cells Following
Exposure Over 48 Hours

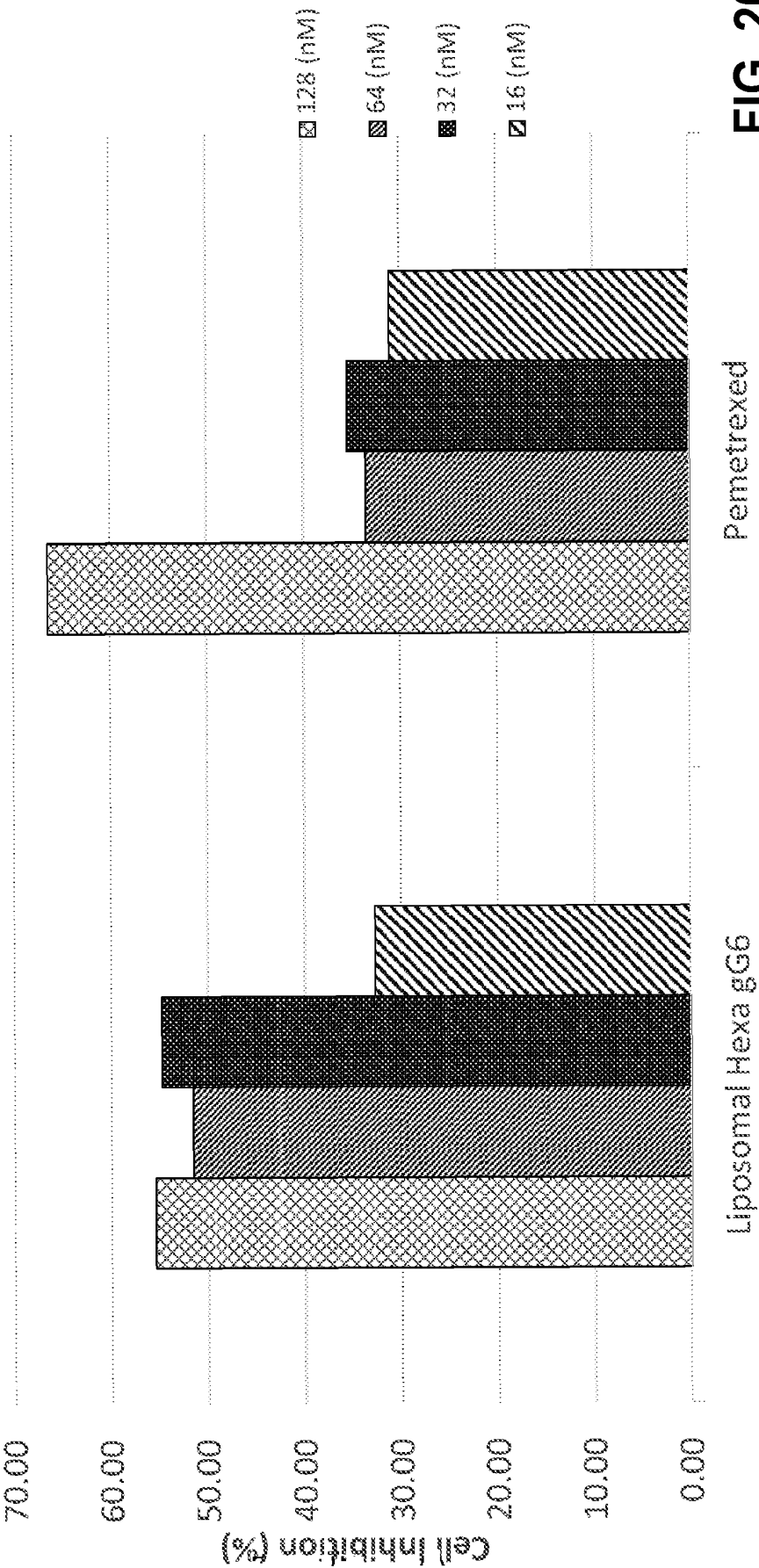


FIG. 20

Treatment Effect on Neutrophils Following Exposure Over 48 Hours

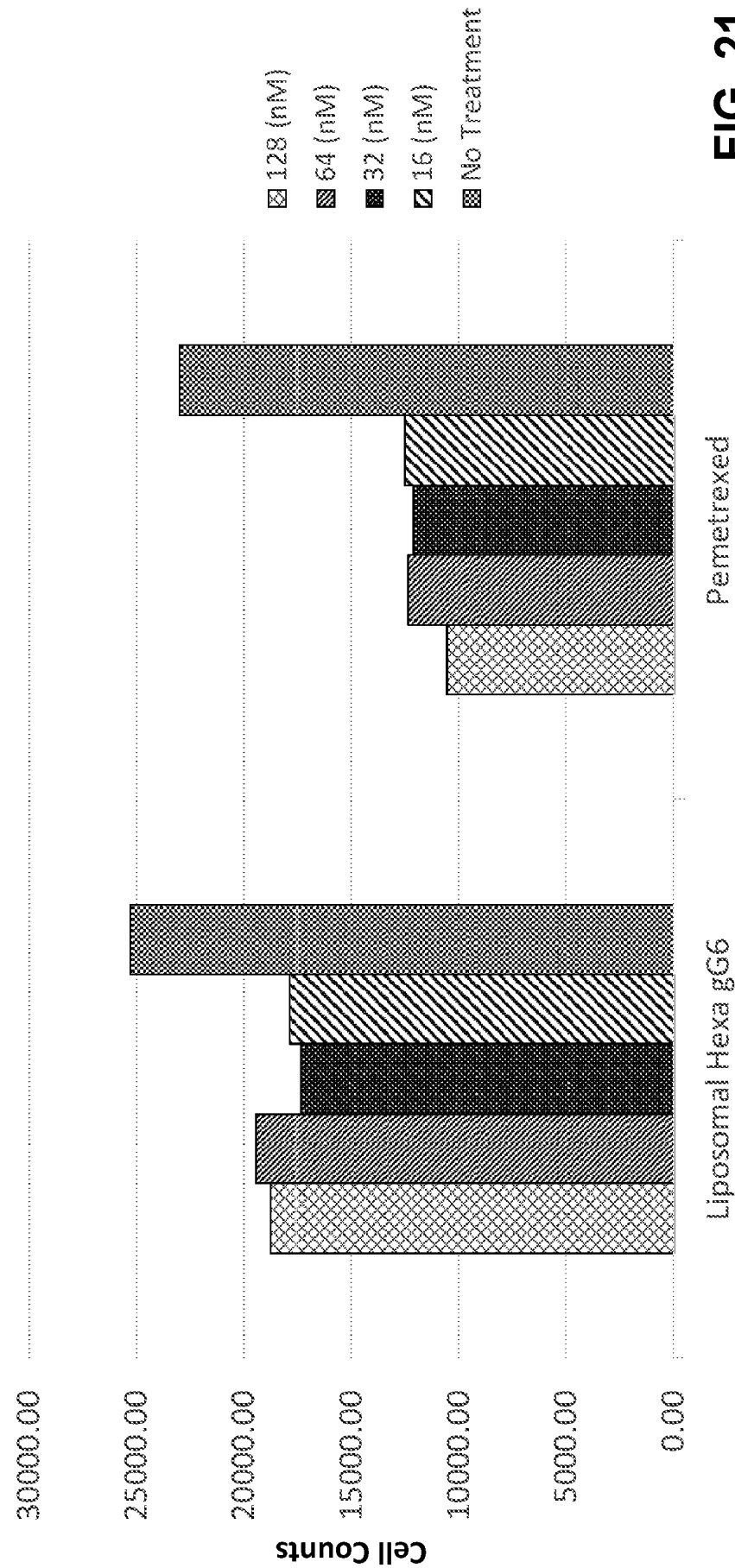


FIG. 21

Treatment Effect on Liver Cells Following Exposure Over 48 Hours

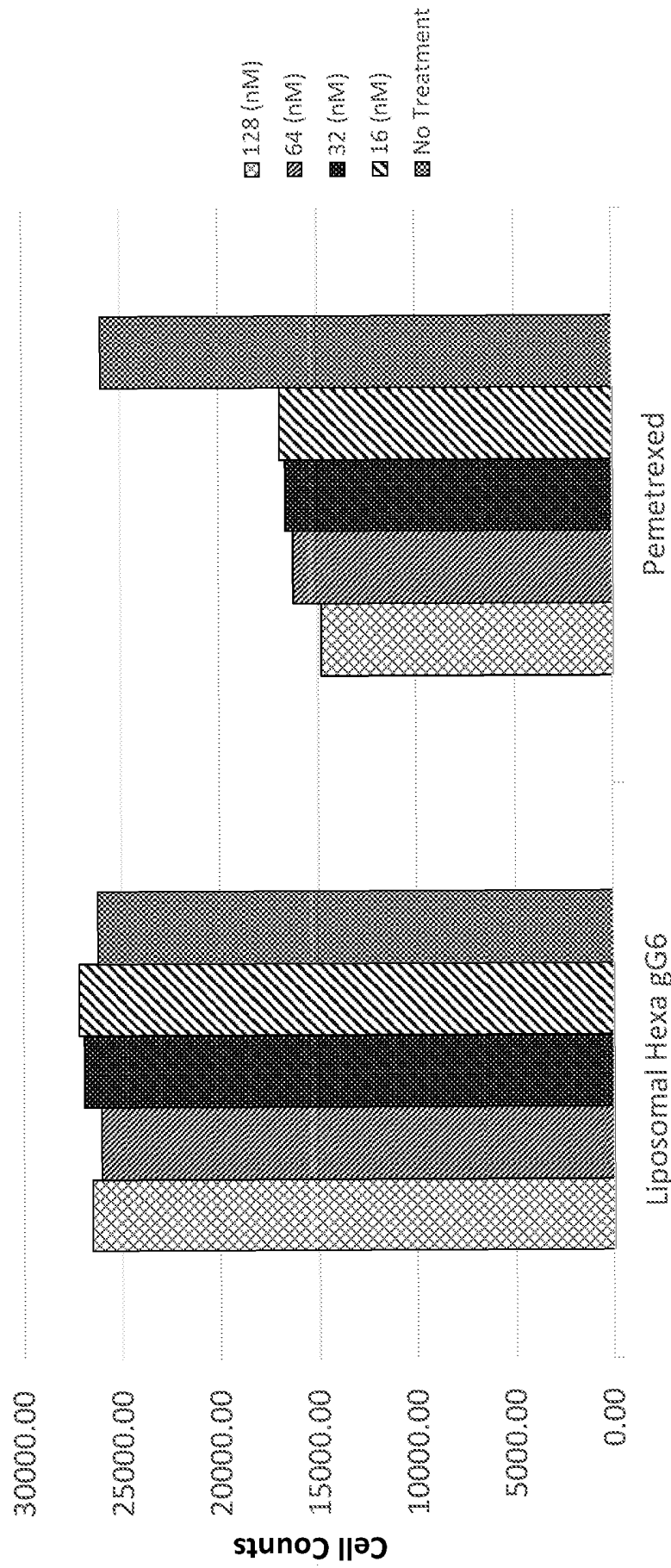


FIG. 22

Treatment Effect on Epithelial Colon Cells Following Exposure Over 48 Hours

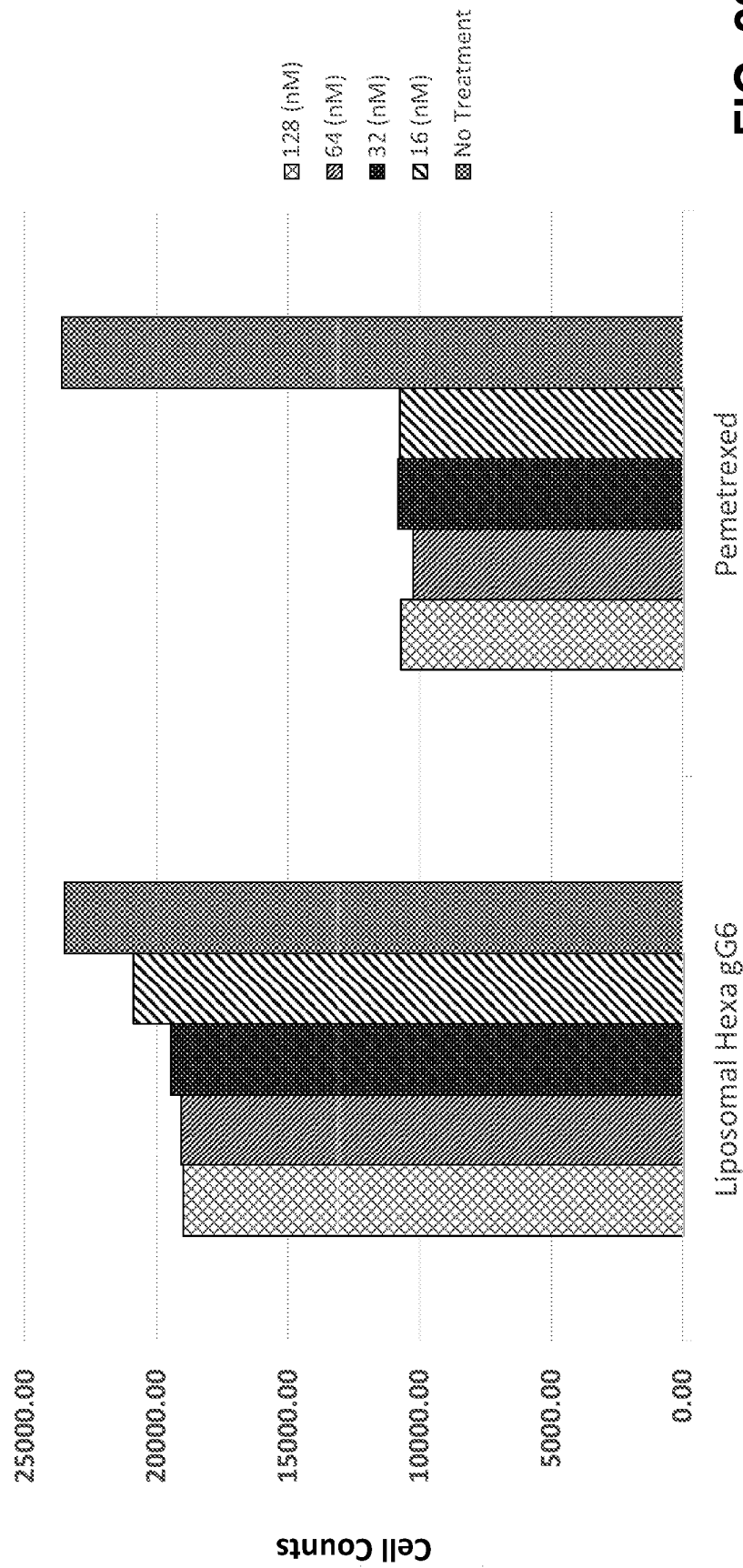


FIG. 23

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 201625882 A [0007] [0056]
- WO 0105405 A [0007]
- WO 2006002049 A [0007]
- WO 2007098089 A [0007]
- WO 2005070465 A [0007]
- WO 2006074416 A [0007]
- US 5225539 A [0147]
- US 5639641 A [0147]
- US 5646253 A [0149]
- US 8388972 B [0149]
- US 8871206 B [0149]
- US 9133275 B [0149]
- US 2011056966 W [0149]
- US 2012046672 W [0149]

Non-patent literature cited in the description

- **MEESTERS et al.** *RAPID COMMUNICATIONS IN MASS SPECTROMETRY*, 2011, vol. 25, 3063-3070 [0007]
- **JACKMAN et al.** *CANCER RESEARCH*, 1991, 5579-5586 [0007]
- **PITTS et al.** *DRUG METABOLISM AND DRUG INTERACTIONS*, 2000, vol. 16, 99-121 [0007]
- **GONEN et al.** *DRUG RESISTANCE UPDATES*, 2012, vol. 15, 183-210 [0007]
- **NG et al.** *BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - BIOMEMBRANES*, 1989, vol. 981, 261-268 [0007]
- **ELDIN et al.** *EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES*, 2015, vol. 81, 60-66 [0007]
- **GONEN et al.** *Drug Resistance Updates*, 2012, vol. 15, 183-210 [0033]
- **JONES et al.** *Nature*, 1986, vol. 321, 522-525 [0147]
- **RIECHMANN et al.** *Nature*, 1988, vol. 332, 323-327 [0147]
- **VERHOEYEN et al.** *Science*, 1988, vol. 239, 1534-1536 [0147]
- *Handbook of Anticancer Drug Development*. LWW, 2003 [0160]
- **SAITO et al.** *Cancer Research*, 2004, vol. 64, 2572-2579 [0170]
- **MAMOT et al.** *J. Neuro-Oncology*, 2004, vol. 68, 1-9 [0170]
- *Liposome Technology*. 1983, vol. 1-3 [0197]
- *Liposome Technology*. CRC Press, 1993 [0197]